

22 DEC 2004

519,128

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
20 November 2003 (20.11.2003)

PCT

(10) International Publication Number  
**WO 03/094697 A2**

(51) International Patent Classification<sup>7</sup>:

A61B

(74) Agent: FRANCIS, Carol, L.; Bozicevic, Field & Francis LLP, Suite 200, 200 Middlefield Road, Menlo Park, CA 94025 (US).

(21) International Application Number: PCT/US03/14304

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(22) International Filing Date: 7 May 2003 (07.05.2003)

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(25) Filing Language: English

**Published:**

(26) Publication Language: English

— without international search report and to be republished upon receipt of that report

(30) Priority Data:

60/379,140	8 May 2002 (08.05.2002)	US
10/291,202	7 November 2002 (07.11.2002)	US
PCT/US02/35932	7 November 2002 (07.11.2002)	US

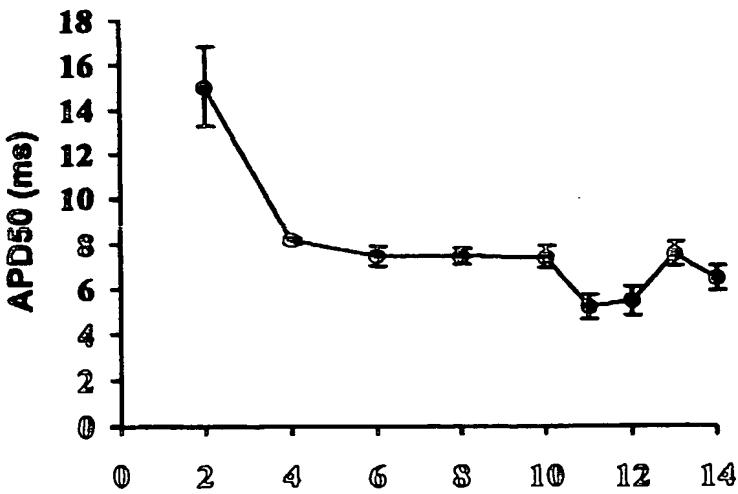
(71) Applicant (*for all designated States except US*): THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 1111 Franklin Street, 12th Floor, Oakland, CA 94604-5200 (US).

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(72) Inventor; and

(75) Inventor/Applicant (*for US only*): LEE, Randall, J. [US/US]; University of California, Cardiac Electrophysiology, Rm. MU429, 500 Parnassus Avenue, San Francisco, CA 94143 (US).

(54) Title: METHODS AND COMPOSITIONS FOR CORRECTION OF CARDIAC CONDUCTION DISTURBANCES



(57) Abstract: The invention provides methods for establishing electrical coupling between cardiomyocytes and recombinant cells which have been genetically engineered to express a gap junction protein, e.g., a connexin protein such as connexin 43 (Cx43) protein. The invention is based on the discovery that genetic modification of skeletal muscle cells to express a recombinant connexin, enables the genetically modified cells to establish electrocommunication with cardiac cells via gap junctions. The recombinant connexin-expressing cells can be used for repair of cardiac tissue and for treatment of cardiac disease by transplantation into cardiac tissue.

BEST AVAILABLE COPY

WO 03/094697 A2

**METHODS AND COMPOSITIONS FOR CORRECTION OF  
CARDIAC CONDUCTION DISTURBANCES**

**STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH**

5        This invention was made with government support under grant nos. DK47766 awarded by the NIH. The government may have certain rights in this invention.

**FIELD OF THE INVENTION**

10      The invention relates generally to the field of treatment of cardiac conduction disturbances, more particularly to recombinant cell transplantation to facilitate cardiac tissue replacement or repair.

**BACKGROUND OF THE INVENTION**

15      Cardiac arrhythmias are a leading cause of morbidity in the Western hemisphere. The risk of developing malignant ventricular tachyarrhythmias is associated with the extent of myocardial injury and is believed to be the primary cause of approximately 50% of all cardiovascular deaths (Myerburg RJ, Kessler KM, Castellanos A., Circulation Jan, (85) I suppl:I2-10, 1992.). Bradycardia and heart block, which can result from the normal aging process, further adds to the morbidity associated with cardiac arrhythmias and results in the permanent implantation of over 160,000 pacemakers annually in the United States.

20      Conventional medical therapy is predominantly palliative treatment and commonly fails to impede and prevent the morbidity and mortality associated with cardiac arrhythmias. Radiofrequency catheter ablation of ischemic ventricular tachycardias is considered adjuvant therapy rather than curative. The implantation of defibrillators and pacemakers, while generally effective, does have problems which include: (1) implantation of a mechanical device and its need for replacement every 4 to 7 years, (2) surgical and mechanical complications resulting from the implantation of the device, (3) negative physical and psychological effects of an implanted mechanical device and (4) a prevalent need to use concurrent antiarrhythmic therapy and/or radiofrequency modulation/ablation.

25      In some instances, and especially where the conduction disturbances are due to ischemia, only more radical options are available, such as surgery. However, even surgical techniques can fall well short of the therapeutic goal of restoring cardiac function in the patient. For example, coronary bypass surgery is frequently inadequate to restore function in patients who have few viable surviving myocytes in the infarct region. Therefore, there is a

need to develop alternative therapies for treatment of myocardial dysfunction that overcome the negative aspects of current treatment methods. In contrast to the conventional treatment modalities which attempt to simulate the physiological process of the heart, the application of tissue engineering to correct conduction disturbances would enhance the natural  
5 physiological processes.

Tissue engineering techniques are attractive alternatives to such conventional therapies. Tissue engineering techniques generally involve transplanting cells that can imitate certain cardiac functions into cardiac tissue to effect myocardial repair (Soonpaa, M., Koh GY, Klug MG, Field LJ, Science, 1994. 264: p. 98-101; Orlic D, Kajstura J, Chimenti, 10 S, Jakonluk I, Anderson SM, Li, B, Pickel J, McKay, R, Nadal-Ginard, B, Bodine, D, Leri A, Anversa P, Nature (2001) 410:701-705. Chiu RC-J, Zibaitis A, Kao RL, Ann Thorac Surg (1995) 60:12-18).

Tissue engineering techniques involving, for example, transplantation of skeletal myoblasts to effect myocardial repair have gained increased attention with the demonstration 15 that skeletal myoblasts survive and form contractile myofibers in normal and injured myocardium (Weisel RD et. al., J. Thoracic Cardiovascular Surgery 2001, 121:835-836; Murry, C., Wiseman RW, Schwartz SM, Hauschka SD, J Clin Invest, 1996. 98: p. 1512-2523; Murry CE, Wiseman RW, Schwartz SM, Hauschka S., J Clin Invest (1996) 98:2512-2523). Cell transplantation and tissue engineering of skeletal myoblast, and stem cells offer 20 the promise of restoring function to patients with limited available myocytes. However, the emphasis of myocardial repair to date has focused on the preservation of myocardial contractility with little attention given to the effects of tissue engineering on cardiac conduction. One concern with the use of skeletal myoblasts transplantation for myocardial repair is whether the skeletal myoblasts will propagate electrical activity to cardiomyocytes.  
25

Cardiomyocytes are electromechanically coupled by intercalated disks composed of adherens and gap junctions. N-cadherin is the major adherens junction protein, whereas connexin 43 (Cx43) is the major gap junction protein in the ventricular myocardium (Verheule S et. al., Circ. Res. 1997, 80:673-81). Due to the difference of cellular electrophysiological properties of cardiac cells and skeletal muscle cells, tight coupling of 30 cardiac and skeletal muscle cells are required for synchronized electrical communication (Lee et al., Annals of Biomedical Engineering 28-1:S54, 2000).

Skeletal myoblasts express N-cadherin and connexin 43 as replicating myoblasts and then downregulate the expression of these two proteins after differentiation and myotube formation. Functional gap junctions have been detected during the early stages of skeletal

muscle development, and gap junction intracellular communication has been suggested to play an important role in myoblast fusion and differentiation (MacCalman, C.D. et. al., Dev. Dyn. 1992, 195:127-132). Although multiple studies have shown that skeletal myoblasts survive cardiac grafting and form myotubes, these studies have not shown whether skeletal 5 fibers form functional junctions with the surrounding cardiomyocytes allowing for electrical communication between the host and grafted cells. Most of these studies have indicated that connexin 43 (Cx43) and N-cadherin are not detectable in the skeletal muscle cells grafted into the host myocardium after cellular differentiation (myotube formation) by the lack of electromechanical coupling between grafted cells and myocardial cells (Murry CE et. al., J. 10 Clin. Invest. 1996, 98:2512-2217; Robinson et. al., Cell Transplantation 1996, 5(1) 77-91; MacCalman, C.D. et. al., Dev. Dyn. 1992 195:127-132; Knudsen, KA et. al., Exp. Cell Res. 1990, 188:175-184; Balogh, S. et. al., Dev. Biol. 1993, 155:351-360; Dahl, E. et. al., Anat. Embryol. 1995, 191:267-278). Previous attempts to transplant skeletal muscle cells into 15 myocardium have lacked the electrical coupling to cardiac cells which is necessary for myocardial coordinated activity.

When skeletal myoblasts and cardiomyocytes, or myotubes and cardiomyocytes, are co-cultured *in vitro*, the cells were found to be electromechanically coupled (Reinecke, H. et. al, J. Cell Biology, 2000, 149(3), 731-740). Reinecke et al. reported that cardiomyocytes were capable of forming electromechanical junctions with some skeletal myotubes *in vitro* 20 and induced their synchronous contraction via gap junctions. N-cadherin and connexin 43 were both detected at the contact sites between cardiomyocytes and skeletal myotubes in this *in vitro* study, although the roles or importance of these proteins, or the mechanism involved, in forming gap junctions remained un-determined. While these studies exemplify the association of connexin 43 expression and functional gap junctions with cardiomyocytes *in* 25 *vitro*, no evidence is presented which indicates that adult skeletal myocytes, which have minimal Cx43 expression, would be capable of forming functional gap junctions in cardiac tissue.

Accordingly, there is a need in the field to provide methods and compositions for induction and enhancement of the electrical coupling between cardiomyocytes and 30 transplanted cells, such as adult skeletal muscle cells, to effect cardiac repair.

#### SUMMARY OF THE INVENTION

The invention provides methods for establishing electrical coupling between cardiomyocytes and recombinant cells which have been genetically engineered to express a

gap junction protein, e.g., a connexin protein such as connexin 43 (Cx43) protein. The invention is based on the discovery that genetic modification of skeletal muscle cells to express a recombinant connexin, enables the genetically modified cells to establish electrocommunication with cardiac cells via gap junctions. The recombinant connexin-expressing cells can be used for repair of cardiac tissue and for treatment of cardiac disease by transplantation into cardiac tissue.

In one aspect the invention features a method of establishing an electrical connection between a recombinant mammalian cell and a myocardial cell, the method comprising contacting a myocardial cell with a recombinant mammalian cell genetically modified to produce a connexin protein, wherein contacting of the cells is in a manner sufficient to provide for production of an electrical connection between the myocardial cell and the recombinant cell. In specific embodiments, the recombinant cell is a skeletal muscle cell, a stem cell, a fibroblast, or a cardiac cell. In an embodiment of interest, the recombinant cell is a skeletal muscle cell, particularly an adult skeletal muscle cell or a myoblast cell. In embodiments of particular interest, the connexin protein is a connexin 43 protein.

In further embodiments, contacting involves implanting the recombinant cell into myocardial tissue of a subject. In further specific embodiments, the electrical connection between the recombinant cell and the myocardial cell is established, the recombinant cell has similar conductive characteristics similar to the myocardial cell.

In another aspect, the invention features a method of establishing an electrical connection between a recombinant skeletal muscle cell and a myocardial cell, the method comprising contacting a myocardial cell with a recombinant skeletal muscle cell genetically modified to express a recombinant connexin protein, where contacting is in a manner sufficient to provide for production of an electrical connection between the myocardial cell and the recombinant skeletal muscle cell. In specific embodiments, the skeletal muscle cell is an adult skeletal muscle cell or a skeletal myoblast cell. In still further embodiments, the electrical connection between the recombinant cell and the myocardial cell is established, the recombinant cell has similar conductive characteristics as the myocardial cell.

In still another aspect, the invention features a method of establishing an electrical connection between a recombinant skeletal muscle cell and a myocardial cell, the method comprising contacting a myocardial cell with a recombinant skeletal myoblast cell genetically modified to express a recombinant connexin 43 protein, wherein contacting is in a manner sufficient to provide for production of an electrical connection between the

myocardial cell and the recombinant skeletal myoblast cell and the recombinant skeletal myoblast cell has similar conductive characteristics as the myocardial cell.

In another aspect the invention features a method for treating a cardiac conduction disturbance in a host, the method comprising introducing into cardiac tissue of a host a therapeutically effective amount of a recombinant mammalian cell, which recombinant cell is genetically modified to express a connexin protein, where introducing is effective to establish an electrical connection between the recombinant cell and a myocardial cell of the host cardiac tissue. In specific embodiments, the recombinant cell is a skeletal muscle cell, a stem cell, a fibroblast, or a cardiac cell. Skeletal muscle cells, particularly an adult skeletal muscle cell or a myoblast cell are of particular interest. In still further embodiments, the connexin protein is a connexin 43 protein. In another embodiment, the recombinant cell is autologous to the host being treated. In related embodiments, introducing is accomplished by implanting the recombinant cell into an infarct region of the cardiac tissue.

In another aspect, the invention features a method for treating a cardiac conduction disturbance in a mammalian host, the method comprising introducing into cardiac tissue of the host a therapeutically effective amount of a skeletal muscle cell genetically modified to express a recombinant connexin 43 protein, where introducing is effective to establish an electrical connection between the introduced recombinant skeletal muscle cell and a myocardial cell of the host cardiac tissue, thereby treating the cardiac conduction disturbance. In specific embodiments, the skeletal muscle cell is an adult skeletal muscle cell or a myoblast cell. In related embodiments, introducing is accomplished by implanting the recombinant cell into an infarct region of the cardiac tissue. In a specific embodiment, the recombinant skeletal muscle cell is autologous to the host.

An aspect of the invention is a method of establishing an electrical connection between a recombinant transplanted cell and a myocardial cell. The method generally involves contacting a cell which has been genetically modified to produce a connexin protein, with a myocardial cell, in a manner that provides for production of an electrical connection between the myocardial cell and the genetically modified cell. In one embodiment of particular interest, the connexin protein is connexin 43.

In certain embodiments, the method generally involves contacting a recombinant mammalian cell genetically modified to produce a connexin protein (such as connexin 43), with a myocardial cell, in a manner that provides for production of a recombinant connexin (e.g. express or overexpress) connection between the myocardial cell and the recombinant

cell, such that an electrical connection between the recombinant cell and the myocardial cell is established. In embodiments of particular interest, the contacting step involves implanting the recombinant cell into myocardial tissue of a subject *in vivo*. In certain embodiments, the recombinant cell is a skeletal muscle cell or myoblast cell, and in particular an adult skeletal muscle cell. The recombinant connexin protein (e. g. connexin 43) is by the recombinant cell in sufficient amounts to induce and/or maintain an electrical connection between the recombinant cell and the myocardial cell. In certain embodiments the recombinant cell has similar conductive characteristics as the myocardial cell after the electrical connection between the recombinant cell and the myocardial cell is established. In one embodiment of particular interest, the connexin protein is connexin 43.

In another aspect, the invention features a method for improving conduction in cardiac tissue (e.g., so as treat cardiac disease) comprising introducing into cardiac tissue of a host a mammalian cell genetically modified to express a connexin protein, the introducing being effective to establish an electrical connection between the introduced genetically modified cell and a myocardial cell of the cardiac tissue, wherein conduction in the cardiac tissue is decreased. In certain embodiments, the method involves introducing a skeletal muscle cell genetically modified to express a connexin 43 protein. In specific embodiments the skeletal muscle cell is an adult skeletal muscle cell or a myoblast cell. In embodiments of particular interest, the host is human. In many instances, the introduction of a recombinant cell comprises surgically implanting the cell into an infarct region of the cardiac tissue for myocardial repair. In certain embodiments, the genetically modified cell originates from a biopsy from the host and the biopsy cell is genetically modified *in vitro* to express a recombinant connexin. In other embodiments the genetically modified cell is from a cell line which has been genetically modified *in vitro* to express a recombinant connexin protein.

These and other aspects, advantages and features of the present invention will become apparent to those persons skilled in the art upon reading the details of the invention more fully set forth below. The invention will now be described in further detail.

#### BRIEF DESCRIPTIONS OF THE DRAWINGS

Figures 1A-1D are graphical representations of the action potential parameters during different periods of myoblasts differentiation to myotubes. FIG. 1A: resting membrane potential (RMP) over 14 days; FIG. 1B: action potential amplitude (APA) over 14 days; FIG. 1C: maximum upstroke velocity (Vmax) over 14 days; FIG. 1D: action potential duration at 50 % (APD<sub>50</sub>) repolarization over 14 days.

Figure 2A is a photograph of an electrophoresis agarose gel of mRNA Cx43 RT-PCR experiments of control cells (TR/Z) and recombinant Cx43-expressing cells at day 0, 2, 4 and 7.

5 Figure 2B is a graphical representation of the average level of Cx43 mRNA determined by RT-PCR for three control samples and three recombinant Cx43-expressing cell samples at day 0, 2, 4 and 7.

Figure 2C is a photograph of a western blot for Cx43 protein, which indicates the relative amounts of Cx43 protein present in control cells and recombinant Cx43-expressing cells at day 0, 2, 4 and 7.

10 Figure 2D is a graphical representation of Cx43 western blotting experiments to determine the relative amount of Cx43 protein in three control cell samples and three Cx43 expressing cell samples at day 0, 2, 4 and 7.

15 Figure 3 is a series of photographs showing the results of a microinjection study between skeletal myoblasts or myotubes indicating the relative transfer of Rhodamine or Lucifer yellow dyes. Row A: control myoblasts to control myoblasts; row B: Cx43 myoblasts to Cx43 myoblasts; row C: control myotube to control myotube; row D: Cx43 abnormal myotube to Cx43 abnormal myotubes.

20 Figure 4 is a series of photographs showing the results of a microinjection study between adult rat cardiomyocytes (ARC) and skeletal myoblasts or myotubes. Injected cells were labeled with Rhodamine and Lucifer Yellow. Row A: ARC to control myoblasts; row B: ARC to recombinant Cx43-expressing cells; row C: ARC to control myotubes; row D: ARC to recombinant Cx43-expressing abnormal myotubes.

25 Figure 5 is a series of photographs showing the results from an Immunofluorescence study analyzing the expression levels of MHC and Desmin, two strong markers for myoblast differentiation into myotubes, in control and Cx43 cells

Before the present invention is described, it is to be understood that this invention is not limited to the particular methodology, protocols, cell lines, vectors and reagents described as such may, of course, vary. It is also to be understood that the terminology used 30 herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and functional

equivalents thereof, and reference to "the polynucleotide" includes reference to one or more polynucleotides and equivalents thereof known to those skilled in the art, and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. Although any methods, devices and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods, devices and materials are now described.

All publications mentioned herein are incorporated by reference for the purpose of describing and disclosing, for example, the cell lines, vectors, and methodologies which are described in the publications which might be used in connection with the presently described invention. The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

15

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention is based upon the discovery that contacting a myocardial cell with a recombinant cell, such as an adult skeletal muscle cell, which is modified to express a recombinant gap junction protein, exemplified by the connexin protein connexin 43 (e.g., in the presence or absence of endogenous connexin 43 expression) allows for electrical coupling of the modified skeletal muscle cell to the myocardial cell. The present invention thus provides methods for using a recombinant cell genetically modified to produce a connexin protein to produce persistent functional gap junctions between the recombinant cell and cardiomyocyte to obtain electrical communication between these cells. The use of recombinant cells that express recombinant Cx43 (or other connexin protein) increases and maintains the communication between the recombinant cells and myocardial cells, thus providing improved and coordinated electrical coupling with increased efficacy of myocardial contractility. The present invention provides methods of treatment of cardiac disease by transplanting or grafting recombinant cells modified to express a connexin into cardiac tissue to effect myocardial repair. Congestive heart failure is an exemplary cardiac disease that can be treated according to the methods of the invention.

#### Definitions

"Polynucleotide" as used herein refers to an oligonucleotide, nucleotide, and fragments or portions thereof, as well as to peptide nucleic acids (PNA), fragments, portions

or antisense molecules thereof, and to DNA or RNA of genomic or synthetic origin which can be single- or double-stranded, and represent the sense or antisense strand. Where "polynucleotide" is used to refer to a specific polynucleotide sequence (*e.g.* a connexin 43 polypeptide-encoding polynucleotide), "polynucleotide" is meant to encompass 5 polynucleotides that encode a polypeptide that is functionally equivalent to the recited polypeptide, *e.g.*, polynucleotides that are degenerate variants (i.e., polynucleotides that encode the same amino acid sequence but differ in polynucleotide sequence due to the degeneracy of the genetic code), or polynucleotides that encode biologically active variants or fragments of the recited polypeptide, including polynucleotides having substantial 10 sequence similarity or sequence identity relative to the sequences provided herein. Similarly, "polypeptide" as used herein refers to an oligopeptide, peptide, or protein. Where "polypeptide" is recited herein to refer to an amino acid sequence of a naturally-occurring protein molecule, "polypeptide" and like terms are not meant to limit the amino acid sequence to the complete, native amino acid sequence associated with the recited protein 15 molecule, but instead is meant to also encompass biologically active variants or fragments, including polypeptides having substantial sequence similarity or sequence identify relative to the amino acid sequences provided herein.

As used herein, " polypeptide" refers to an amino acid sequence of a recombinant or non-recombinant polypeptide having an amino acid sequence of i) a native polypeptide, ii) a 20 biologically active fragment of an polypeptide, iii) biologically active polypeptide analogs of an polypeptide, or iv) a biologically active variant of an polypeptide. Polypeptides useful in the invention can be obtained from any species, *e.g.*, mammalian or non-mammalian (*e.g.*, reptiles, amphibians, avian (*e.g.*, chicken)), particularly mammalian, including human, rodenti (*e.g.*, murine or rat), bovine, ovine, porcine, murine, or equine, preferably rat or 25 human, from any source whether natural, synthetic, semi-synthetic or recombinant. For example, "Human connexin 43 polypeptide" refers to the amino acid sequences of isolated human Cx43 polypeptide obtained from a human, and is meant to include all naturally-occurring allelic variants, and is not meant to limit the amino acid sequence to the complete, native amino acid sequence associated with the recited protein molecule.

30 A "variant" of a polypeptide is defined as an amino acid sequence that is altered by one or more amino acids (*e.g.*, by deletion, addition, insertion and/or substitution). Generally, "addition" refers to nucleotide or amino acid residues added to an end of the molecule, while "insertion" refers to nucleotide or amino acid residues between residues of a naturally-occurring molecule. The variant can have "conservative" changes, wherein a

substituted amino acid has similar structural or chemical properties, e.g., replacement of leucine with isoleucine. More rarely, a variant can have "nonconservative" changes, e.g., replacement of a glycine with a tryptophan. Similar minor variations can also include amino acid deletions or insertions, or both. Guidance in determining which and how many amino acid residues may be substituted, added, inserted or deleted without abolishing biological or immunological activity can be found using computer programs well known in the art, for example, DNASTar software.

By "nucleic acid of interest" is meant any nucleic acid (e.g., DNA) which encodes a protein or other molecule which is desirable for inducing or maintaining electrical coupling between cells. In general, the nucleic acid is operatively linked to other sequences which are needed for its regulation and expression, such as a promoter and regulatory elements.

The term "biologically active" refers to, for example, a human connexin polypeptide having structural, regulatory, or biochemical functions of a naturally occurring connexin polypeptide, particularly with respect to facilitating the establishment of an electrochemical connection between a cell modified to express a connexin polypeptide and a myocardial cell. Likewise, "immunologically active" defines the capability of the natural, recombinant or synthetic human connexin polypeptide, or any oligopeptide thereof, to induce a specific immune response in appropriate animals or cells and to bind with a connexin specific antibody.

The term "derivative" as used herein refers to the chemical modification of a nucleic acid encoding a polypeptide or the encoded polypeptide. Illustrative of such modifications would be replacement of hydrogen by an alkyl, acyl, or amino group. A nucleic acid derivative would encode a polypeptide which retains essential biological characteristics of a natural polypeptide.

As used herein the term "isolated" is meant to describe a compound of interest (e.g., either a polynucleotide or a polypeptide) that is in an environment different from that in which the compound naturally occurs. "Isolated" is meant to include compounds that are within samples that are substantially enriched for the compound of interest and/or in which the compound of interest is partially or substantially purified.

As used herein, the term "substantially purified" refers to a compound (e.g., either a polynucleotide or a polypeptide) that is removed from its natural environment and is at least 60% free, preferably 75% free, and most preferably 90% free from other components with which it is naturally associated.

By "transformation", "transduction" or "transfection" is meant a permanent or transient genetic change, preferably a permanent genetic change, induced in a cell following incorporation of new nucleic acid (e.g., DNA or RNA exogenous to the cell). Genetic change can be accomplished either by incorporation of the new nucleic acid into the genome 5 of the host cell, or by transient or stable maintenance of the new DNA as an episomal element.

By "transformed cell", "transfected cell" or "transduced cell" is meant a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding a protein of interest.

10 By "construct" is meant a recombinant nucleic acid, generally recombinant DNA, that has been generated for the purpose of the expression of a specific nucleotide sequence(s), or is to be used in the construction of other recombinant nucleotide sequences. Constructs useful in the invention are those which comprise connexin-encoding gene sequence operably linked to a promoter which will allow for the expression of the connexin 15 protein in a transformed cell. Exemplary constructs useful for the expression of human and rat Cx43 in accordance with the invention are described in Shinoura, N, et al., J Neurosurg. 1996 May;84(5):839-45 and Suzuki et al, Ann. Thorac. Surg., 2001, 71:1724-33, respectively.

20 By "promoter" is meant a minimal sequence sufficient to direct transcription in a recombinant cell. "Promoter" is also meant to encompass those elements sufficient for promoter-dependent gene expression controllable for cell-type specific, tissue-specific or inducible by external signals or agents; such elements may be located in the 5' or 3' regions of the native gene (e.g., enhancer elements).

25 By "operably linked" or "operatively linked" is meant that a DNA sequence and a regulatory sequence(s) are connected in such a way as to permit expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequence(s).

30 By "connexin gene" is meant the open reading frame encoding a connexin polypeptide, or introns, or biologically active fragment thereof. "Connexin gene" includes adjacent 5' and 3' non-coding nucleotide sequences involved in the regulation of expression, up to about 10 kb beyond the coding region, but possibly further in either direction. The DNA sequences encoding a connexin may be cDNA or genomic DNA or a fragment thereof. The gene may be introduced into an appropriate vector for extrachromosomal maintenance or for integration into the host.

The term "cDNA" as used herein is intended to include all nucleic acids that share the arrangement of sequence elements found in native mature mRNA species, where sequence elements are exons (e.g., sequences encoding open reading frames of the encoded polypeptide) and 3' and 5' non-coding regions. Normally mRNA species have contiguous 5 exons, with the intervening introns removed by nuclear RNA splicing, to create a continuous open reading frame encoding the polypeptide of interest.

By "cardiomyocyte" is meant a cardiac contractile cell, which is a cardiac muscle cell. The cardiomyocyte cell may be isolated and cultured *in vitro* or be part of the myocardium of a host.

10 By "skeletal muscle cell" is meant a cell found in skeletal muscle which includes but not limited to myoblasts, myotubes and mature skeletal muscle cells.

By "recombinant cell" is meant a cell comprising nucleic acid not normally associated with the cell (e.g. a cell transformed, transduced or transfected with a construct encoding a specific protein, e.g., a connexin protein).

15 By "transplanted cell" is meant a cell which has been introduced into a host so as to be in contact with a cell within a host. For example, a recombinant cell or cells maybe grafted and/or implanted into the cardiac tissue of a host.

By "therapeutically effective amount" in the context of treatment of cardiac conduction disturbances is meant an amount effective to decrease a symptom of cardiac 20 conduction disturbance and/or to improve cardiac conductance (a measure of conduction).

By "overexpressing" or "overexpression" of a gene product (such as a Cx43 protein) is meant an increased level of protein expression over a normal level of protein expression for a particular cell or cell type at, for example, a particular developmental stage or stage of differentiation. In certain instances, overexpressing can be a cumulative effect of protein 25 expression from endogenous and recombinant genes or essentially protein expression from a recombinant gene. Overexpression of a connexin (e.g., Cx43) is meant to refer to the expression of connexin protein within a particular cell which is above the connexin expression level normally associated with a normal or wild-type cell at a particular stage of differentiation. For cells which normally do not express significant or detectable amounts of 30 the connexin (e.g. as with Cx43 in adult skeletal muscle cells or myotubes), overexpression of connexin protein would mean any detectable expression of connexin, and particularly a level of expression sufficient to promote establishment of an electrochemical connection between the recombinant cell in which connexin expression is elevated and a cardiomyocyte. In certain embodiments overexpression of connexin is meant an increase in expression by a

factor of at least about 2 fold, in other embodiments at least about 5 fold and yet in still other embodiments at least about 10 fold.

The terms "subject", "patient", "host" and "individual" are used interchangeably herein to refer to any mammalian subject for whom diagnosis or therapy is desired, particularly humans. Other subjects may include cattle, dogs, cats, guinea pigs, rabbits, rats, mice, horses, and so on. Of particular interest are subjects having a myocardial associated disorder that is amenable to treatment (e.g., to mitigate symptoms associated with the disorder) by the transplantation of cells which express a recombinant connexin (e.g., Cx43) into the subject (e.g., by introduction of a recombinant connexin expressing cell into the subject *in vivo*, or by grafting cells expressing a connexin (e.g., adult skeletal myoblasts, stem cells (e.g., mesenchymal, hematopoietic), fibroblasts, cardiac cells, etc.) into the subject. In many embodiments the hosts will be humans.

By "electrical coupling" is meant the interaction between cells which allows for intracellular communication between cells so as to provide for electrical conduction between the cells. Electrical coupling *in vivo* provides the basis for, and is generally accompanied by, electromechanical coupling, in which electrical excitation of cells through gap junctions in the muscle leads to muscle contraction.

By "cardiac conduction disturbance" is meant a disturbance in the normal generation and transmission of the electrical activity that initiates myocardial contraction. Cardiac arrhythmias resulting from electrical conduction disturbances can lead to life threatening ventricular tachyarrhythmias, hemodynamically compromising bradycardias, and heart block.

By "condition related to a cardiac conduction disturbance" is meant a condition, symptom or disorder associated with cardiac conduction disturbance. Examples of conditions related to cardiac conduction disturbance are irregular heart beat, fatigue, shortness of breath, and lack of synchronized heart muscle contraction.

By "treatment", "treating", or "treat" is meant that at least an amelioration of the symptoms associated with the condition afflicting the host is achieved, where amelioration is used in a broad sense to refer to at least a reduction in the magnitude of a parameter, e.g. symptom (such as irregular heart beat, fatigue, shortness of breath, syncope can be symptoms associated with conduction disturbances as heart block, ventricular tachycardias or associated with congestive heart failure (i.e. lack of synchronized contraction)) associated with the condition being treated. As such, treatment also includes situations where the pathological condition, or at least symptoms associated therewith, are completely inhibited,

e.g. prevented from happening, or stopped, e.g. terminated, such that the host no longer suffers from the condition, or at least the symptoms that characterize the condition.

**METHODS OF ESTABLISHING ELECTRICAL CONNECTION BETWEEN A CONNEXIN-OVEREXPRESSING CELL AND A MYOCARDIAL CELL**

5

The present invention provides methods for establishing an electrical connection between a recombinant cell expressing a connexin, and a myocardial cell. The methods generally involve contacting a connexin recombinant cell (e.g., a skeletal muscle cell, stem cell (e.g., mesenchymal, hematopoetic), fibroblast, cardiac cell, etc.) with a myocardial cell in 10 a manner that provides for production of an electrical connection between the myocardial cell and the recombinant cell. The cell is recombinant, e.g., it is genetically modified to produce a biologically active connexin protein, e.g., connexin 43 (Cx43) protein. Production of connexin in the recombinant cell provides for an electrical connection, and thus an electromechanical connection, between the recombinant cell and the myocardial cell.

15

***Connexin-Encoding Nucleic Acids***

As summarized above, the methods of the invention utilize nucleic acid compositions, including genomic and cDNA nucleic acid compositions, that encode biologically active connexin 43 proteins, or biologically active fragments, homologs, or analogues thereof suitable for expression in a recombinant cell which cell can subsequently 20 form a electrochemical connection with a cardiac cell.

By "connexin protein" is meant a protein from the family of homologous proteins found in connexins of gap junctions as homo- or heterohexameric arrays. Connexin proteins are the major gap junction protein involved in the electrical coupling of cells. Gap junctions regulate intercellular passage of molecules, including inorganic ions and second messengers, 25 thus achieving electrical coupling of cells. Over 15 connexin subunit isoforms are known, varying in size between about 25 kDa and 60 kDa and generally having four putative transmembrane  $\alpha$ -helical spanners. Different connexins are specific for various parts of the heart. Connexin family proteins found in the cardiovascular system includes Cx37, Cx40, Cx43, and Cx45 (van Veen, AA; van Rijen, HV; Ophof, T., *Cardiovascular Research* 2001 Aug 1, 51(2):217-29.; Severs, NJ; Rothery, S; Dupont, E; Coppen, SR; Yeh, HI; Ko, YS; Matsushita, T; Kaba, R; Halliday, D., *Microscopy Research and Technique* 2001 Feb 1, 52(3):301-22; Kwong, KF; Schuessler, RB; Green, KG; Laing, JG; Beyer, EC; Boineau, JP; Saffitz, JE., *Circulation Research* 1998 Mar 23, 82(5):604-12).

As used interchangeably herein, "Connexin 43" and "Cx43" refer to the amino acid sequences of an isolated Cx43 polypeptide, having structural, regulatory, or biochemical functions associated with gap junctions and electromechanical coupling, obtained from any species, particularly mammalian, including human, rodent (e.g., murine or rat), bovine, ovine, porcine, murine, or equine, preferably human, and may be natural, synthetic, semi-synthetic or recombinant, and is meant to include all naturally-occurring allelic variants, and is not meant to limit the amino acid sequence to the complete, native amino acid sequence associated with the recited protein molecule. Cx43 encompasses biologically active Cx43 fragments. Examples of Cx43 include human Cx43 (Genbank Accession Nos. XP\_027460, XP\_027459, XP\_004121, P17302, AAD37802, A35853, NP\_000156, AF151980, M65188, and AAA52131), mouse Cx43 (Genbank Accession Nos. P23242, P18246, A39802, A36623, NP\_034418, NM\_012567, NM\_010288, CAA44640) and rat Cx43 are found at Genbank Accession Nos. P08050, S00532, NP\_036699, AAA75194 and 1404339A.

A connexin genomic sequence of interest comprises the nucleic acid present between the initiation codon and the stop codon, with a connexin 43 gene being of particular interest, including all of the introns that are normally present in a native chromosome. It may further include the 3' and 5' untranslated regions found in the mature mRNA. It may further include specific transcriptional and translational regulatory sequences, such as promoters, enhancers, *etc.*, including about 10 kb, but possibly more, of flanking genomic DNA at either the 5' or 3' end of the transcribed region. The genomic DNA may be isolated as a large fragment of 100 kbp or more, or as a smaller fragment substantially free of flanking chromosomal sequence. In another embodiment, the connexin DNA is a cDNA, which lacks intronic sequences that may be found in the genomic DNA. The cDNA may be operably linked to a promoter that is normally associated with the connexin sequence (*e.g.*, a promoter endogenous to the connexin gene) or that is heterologous to the connexin sequence (*i.e.*, a promoter from a source other than the connexin sequence).

The sequence of this 5' region, and further 5' upstream sequences and 3' downstream sequences, may be utilized for promoter elements, including enhancer binding sites, that provide for expression in tissues where the connexin polypeptide is normally expressed. The connexin sequence used can be based on the nucleotide sequences of any species (*e.g.*, mammalian or non-mammalian (*e.g.*, reptiles, amphibians, avian (*e.g.*, chicken))), particularly mammalian, including human, rodent (*e.g.*, murine or rat), bovine, ovine, porcine, murine, or equine, preferably rat or human) and can be isolated or produced from any source whether

natural, synthetic, semi-synthetic or recombinant. Where the recombinant cell is a human cell, or where the cardiac tissue into which the cell is to be implanted is human, the connexin is preferably a human connexin or derived from a human connexin.

The nucleic acid compositions used in the subject invention may encode all or a part, 5 usually at least substantially all, of the connexin polypeptide as appropriate. Fragments may be obtained of the DNA sequence by chemically synthesizing oligonucleotides in accordance with conventional methods, by restriction enzyme digestion, by PCR amplification, *etc.* For the most part, DNA fragments will be of at least about 100 contiguous nucleotides, usually at least about 200 nt, more usually at least about 250 nt to about 500 nt.

10 The connexin genes are isolated and obtained in substantial purity, generally as other than an intact mammalian chromosome. Usually, the DNA will be obtained substantially free of other nucleic acid sequences that do not include a sequence encoding a Cx43 or fragment thereof, generally being at least about 50%, usually at least about 90% pure and are typically "recombinant", *i.e.* flanked by one or more nucleotides with which it is not 15 normally associated on a naturally occurring chromosome.

The sequence of the connexin protein, including flanking promoter regions and coding regions, may be mutated in various ways known in the art to generate targeted changes in promoter strength, sequence of the encoded protein, *etc.* The DNA sequence or product of such a mutation will be substantially similar to one or more of the sequences 20 provided herein, *i.e.* will differ by at least one nucleotide or amino acid, respectively, and may differ by at least two, or by at least about ten or more nucleotides or amino acids. In general, the sequence changes may be additions, substitutions, insertions or deletions. Deletions may further include larger changes, such as deletions of a domain or exon. Such modified connexins sequences can be used, for example, to generate constructs for 25 introduction into cells for the purpose of promoting production of electrochemical connections.

It should be noted that preferably the connexin gene is selected according to the genus and species of the host (*e.g.*, where a human is to receive Cx43-modified cells, then the Cx43 gene sequence is a human Cx43).

30 The encoded connexin is biologically active, *e.g.*, when produced in a skeletal muscle cell, a biologically active Cx43 polypeptide facilitates establishment of a connection between the skeletal muscle cell and a myocardial cell. Without being held to theory, the connexin protein (*e.g.*, Cx43) is expressed at the cell surface and is inserted into the plasma membrane as part of gap junctions. To establish electrical coupling between cells, connexin

must be functional gap junctions to form gap junctional intercellular communication (GJIC). The identification of an electrical connection between two cells (e. g. such as an adult skeletal muscle cell and a myocardial cell) can be readily determined by those skilled in the art. Gap junctions can be evaluated by microinjecting cells with a gap junction permeable dye, e.g., Lucifer yellow (Molecular Probes, Or.), which is transferred from one cell to another when functional gap junctions are present. A micro injection protocol for detecting functional gap junctions (i.e. functional expression of Cx43) is given in the Examples section.

The recombinant cells can optionally be genetically modified to express other proteins, such as N-cadherin protein. However, the cells are preferably not so modified so as to avoid additional genetic manipulation of the cell to be transplanted. Furthermore, the recombinant cell need not be modified to express or overexpress N-cadherin, as the inventors here have shown that expression of an exogenous (e.g., introduced or recombinant) connexin (either in the presence or absence of expression of any endogenous connexin) is sufficient.

#### *Constructs For Connexin Nucleic Acids*

Constructs comprising connexin nucleic acids are well known in the art. For example, constructs containing the connexin 43 gene are described by El Oakley, et al, Ann. Thorac. Surg., 2001, 71:1724-33. Constructs comprising connexin-encoding nucleic acids are utilized to transform, transfet or transduce specific cells of interest to allow for the expression of an introduced connexin-encoding nucleic acid molecule in the modified cell.

Where the nucleic acid to be expressed is DNA, any construct having a promoter (e.g., a promoter that is functional in a eukaryotic cell) operably linked to a DNA of interest can be used in the invention. The constructs containing the DNA sequence (or the corresponding RNA sequence) which may be used in accordance with the invention may be any expression construct suitable for use in a mammalian cell, and containing the DNA or the RNA sequence of interest. Such constructs can include nucleic acid of a plasmid or viral construct (e.g. adeno associated virus, adenovirus, and the liked) and can be circular or linear. Preferably the construct is capable of replication in eukaryotic and/or prokaryotic hosts. Suitable constructs are known in the art and are commercially available. The constructs can be prepared using techniques well known in the art. Likewise, techniques for obtaining expression of exogenous DNA or RNA sequences in a genetically altered host cell are known in the art.

In one embodiment, the DNA construct contains a promoter to facilitate expression of the DNA of interest within a mammalian cell. The promoter may be a strong promoter that functions in mammalian cells, such as a promoter from cytomegalovirus (CMV), mouse mammary tumor virus (MMTV), Rous sarcoma virus (RSV), lenti-virus or adenovirus.

- 5 More specifically, exemplary promoters include the promoter from the immediate early gene of human CMV (Boshart et al., Cell 41:521-530, 1985) and the promoter from the long terminal repeat (LTR) of RSV (Gorman et al., Proc. Natl. Acad. Sci. USA 79:6777-6781, 1982). Alternatively, the promoter used may be a strong general eukaryotic promoter such as the actin gene promoter. In one embodiment, the promoter used may be a tissue-specific 10 promoter. For example, the promoter used in the construct may be a cardiac cell specific promoter, a myoblast specific promoter or an adult skeletal muscle cell specific promoter (Luo, et. al., Development 2001 Feb, 128(4):459-69; Lee, et. al., J. Thor. Card. Sur. 1999 Jul, 118(1):26-4, discussion 34-5). Primary cardiac myocytes from neonatal rats have been transfected with a reporter construct driven by the C promoter of rat acyl-coenzyme 15 synthetase gene (Kanda, et al. Heart Vessels 2000, 15(4):191-6) as well as alpha- and beta-cardiac myosin heavy chain gene promoters(James, et. al., Circulation 2000 Apr 11, 101(14):1715-21).

The constructs of the invention may also include sequences in addition to promoters which enhance and regulate connexin expression in modified cells. For example the serum 20 response factor (SRF) gene has been shown to regulate transcription of numerous muscle and growth factor-inducible genes. Because SRF is not muscle specific, it has been postulated to activate muscle genes by recruiting myogenic accessory factors. Myocardin is a member of a class of muscle transcription factors, provides a mechanism whereby SRF can convey myogenic activity to muscle genes. (Wang, et. al., Cell. 2001 Jun 29;105(7):851-62).

25 In another embodiment, the promoter is a regulated promoter (e.g., inducible promoter), such as a tetracycline-regulated promoter, expression from which can be regulated by exposure to an exogenous substance (e.g., tetracycline). Another example of regulated promoter system useful in the present invention is the lac operator-repressor gene regulatory system to regulate mammalian promoters (Cronin, et. al., Genes Dev. 2001 Jun 30, 15, 15(12):1506-17).

For eukaryotic expression, the construct should contain at a minimum a eukaryotic promoter operably linked to a DNA of interest, which is in turn operably linked to a polyadenylation signal sequence. The polyadenylation signal sequence may be selected from any of a variety of polyadenylation signal sequences known in the art. An exemplary

polyadenylation signal sequence is the SV40 early polyadenylation signal sequence. The construct may also include one or more introns, where appropriate, which can increase levels of expression of the DNA of interest, particularly where the DNA of interest is a cDNA (e.g., contains no introns of the naturally-occurring sequence). Any of a variety of introns known in the art may be used (e.g., the human  $\beta$ -globin intron, which is inserted in the construct at a position 5' to the DNA of interest).

5 In an alternative embodiment, the nucleic acid delivered to the cell is an RNA encoding a connexin protein. In this embodiment, the RNA is adapted for expression (i.e., translation of the RNA) in a target cell. Methods for production of RNA (e.g., mRNA) 10 encoding a protein of interest are well known in the art, and can be readily applied to the product of RNA encoding connexin useful in the present invention.

#### ***Production Of Recombinant Connexin Cells***

Cells to be modified to express a recombinant connexin include any cell capable of coupling with a cardiomyocyte via connexin-mediated gap junctions, including skeletal 15 muscle cells, stem cells (e.g., mesenchymal, hematopoietic), fibroblasts, cardiac cells, and the like, following genetic modification to provide for expression of a recombinant connexin (e.g., Cx43) in the cell. In one embodiment of particular interest, the cells are skeletal muscle cells.

Cells may be obtained from the host (e.g., endogenous cells) or from appropriate 20 cultured cell lines. Cells may be autologous, allogeneic, or xenogeneic (e.g., primate, pig, etc.) with respect to the host. In certain embodiments, the cells are collected from the subject or patient via biopsy (e.g., muscle biopsy). This latter embodiment allows for autologous transplantation of recombinant connexin-expressing cells into host myocardium.

Cells suitable for use to produce recombinant connexin-expressing cells include 25 skeletal muscle cells, particularly adult skeletal muscle cells, stem cells (e.g., mesenchymal, hematopoietic), fibroblasts, cardiac cells, and the like. An expression construct that provides for production of connexin (e.g., Cx43) is then introduced into the cells which may be propagated and cultured *in vitro* before and/or after transformation to increase the number 30 of recombinant connexin-expressing cells available for transplantation into myocardial tissue.

In one embodiment, the cell is a skeletal cell muscle cell or cell line, propagated and transformed with an appropriate vector for the expression of a connexin (e.g., Cx43). These recombinant connexin expressing cells are cultured *in vitro* and utilized for transplantation

into myocardium. In another embodiment, the cells are cells of a fresh primary culture or a frozen culture.

Methods for introducing connexin constructs into a mammalian cell include standard protocols known to those skilled in the art.

5       The regulation of connexin expression can be accomplished using regulatory elements operably inserted into the construct comprising the connexin gene used to transduce the modified cells. Other methods of regulating connexin expression may include genomic regulatory elements endogenous to the recombinant cells or by the addition of compounds that modulate connexin expression (e.g., either at the time of or following  
10      implanting the recombinant cells.)

Connexin expression in the modified cells can be detected by such techniques as western blotting, utilizing antibodies specific for the recombinant connexin. Other methods for confirming the expression of a recombinant connexin in transformed cells may involve RT-PCR utilizing primers specific for connexin mRNA or immunofluorescence techniques  
15      on transformed cells in culture. The ability of a connexin polypeptide, to facilitate production of an electrical connection between a recombinant cell and a cardiomyocyte can be tested in an *in vivo* model.

***Production Of Functional Gap Junctions Between Recombinant Connexin Cells And Cardiomyocytes***

20      The recombinant connexin-expressing cells can be cultured to expand the number of cells *in vitro*. After a desired number of recombinant cells are obtained, the cells are introduced into myocardial tissue. Alternatively or in addition, recombinant connexin cells and myocardial cells are co-cultured *in vitro* and then transplanted.

25      Production of a connexin allows the modified cells to induce an electrical connection with myocardial cells via gap junctions. Due to the difference in the cellular and electrophysiological properties of myocardial cells and non-myocardial cells, tight coupling of myocardial and non-myocardial cells is required for synchronized electrical communication. The present invention demonstrates a unique and novel interaction between two different cell types which allows for the treatment and therapy of myocardial diseases  
30      and disorders.

**METHODS OF TREATING CARDIAC CONDITIONS**

The instant invention provides methods for correction of cardiac conduction disturbances and methods for treating cardiac conditions related to a cardiac conduction

disturbance. The present invention is an advancement over standard cellular transplantation by increasing cell to cell communication, thus allow for more synchronized contraction. The methods generally involve contacting a cardiac tissue of a host with a recombinant cell that expresses a connexin protein (e.g., Cx43), such that the connexin protein facilitates production of an electrical connection between the recombinant cell and the cardiomyocyte. The connection facilitates correction of a cardiac conduction disturbance by improving conduction in the heart. In embodiments of particular interest, the recombinant cell is a skeletal muscle cell.

The subject methods find use in the treatment of a variety of different conditions in which an increase coordinated conduction of cardiomyocytes is desired.

Exemplary diseases amenable to treatment by the methods of the invention include, but are not limited to, complete heart block, reentrant arrhythmias (e.g., ventricular tachycardia) congestive heart failure, and the like. Any cardiac disease or disorder that would benefit from improved synchronized contraction is amenable to treatment with the methods of the present invention.

#### *Implantation Of Recombinant Connexin Cells*

The transplantation of recombinant connexin cells into the myocardium of a subject can use well known surgical techniques for grafting tissue and/or isolated cells into a heart. In general, there are two methods for introducing the recombinant cells into the subject's heart tissue: 1) surgical, direct injection; or 2) percutaneous techniques as describe in US Patent No. 6,059,726 (Lee and Lesh, "Method for locating the AV junction of the heart and injecting active substances therein").

The recombinant connexin cells can be implanted into any area of the heart where conduction disturbances have occurred. The amount of recombinant cells to be transplanted is determined by the type of heart disease being treated, the overall damage of myocardial tissue and the level of connexin expression in the cells to be transplanted.

In certain embodiments, the recombinant connexin-expressing cells are transplanted by percutaneous methods. If the site of the damaged heart tissue can be accurately determined in a subject by non-invasive diagnostic techniques, the recombinant connexin cells can be injected directly into the damaged myocardial tissue using general methods for percutaneous injections into cardiac muscle well known in the art. The amount of recombinant cells necessary to be therapeutically effective will vary with the type of disorder being treated as well as the extent of heart damage that has occurred.

Immunosuppressants may be used in conjunction of transplantation of Cx43-overexpressing cells not derived from the host to minimize the possibility of graft rejection, e.g., allogeneic or xenogeneic cells.

***Combination With Other Therapies***

5       The methods of the subject invention may also be utilized in combination with other cardiac therapies when appropriate. In certain embodiments, drugs used to treat certain types of conduction defects can be administered in combination with implanting recombinant connexin cells into the damaged myocardium (e.g., prior to, during and/or after implantation). Cardiac drugs that are suitable for use in combination therapy with the  
10      methods of the invention include, but are not limited to, growth factors, polynucleotides encoding growth factors, angiogenic agents, calcium channel blockers, antihypertensive agents, antimitotic agents, inotropic agents, antiatherogenic agents, anti-coagulants, beta-blockers, anti-arrhythmic agents, antiinflammatory agents, vasodilators, thrombolytic agents, cardiac glycosides, antibiotics, antiviral agents, antifungal agents, agents that inhibit  
15      protozoans, antiarrhythmic agents (used for treatment of ventricular tachycardia), nitrates, angiotensin converting enzyme (ACE) inhibitors; brain natriuretic peptide (BNP); antineoplastic agents, steroids, and the like.

The present invention may also be a supplemental procedure to coronary artery bypass grafting (CABG). Replacement of a non-functioning myocardial scar with  
20      functioning muscle together with revascularization improves myocardial performance more than revascularization (bypass surgery) alone. Transplantation of recombinant connexin cells in conjunction with CABG provides for additive treatment during surgery by preventing the continued myocardial remodeling by reducing wall stress and ischemic burden. Additional surgical procedures to deliver the recombinant cells into the myocardium  
25      can be avoided by implanting the recombinant cells at the time of CABG surgery.

***Assessment of Therapy***

The effects of therapy according to the methods of the invention can be monitored in a variety of ways. Generally for heart block disorders, an electrocardiogram (ECG) or holter monitor is utilized to determine the efficacy of treatment. The contraction of the heart  
30      occurs due to electrical impulses that are generated within the heart; an ECG is a measure of the heart rhythms and electrical impulses. Thus ECG is a very effective and non-invasive way to determine if therapy has improved or maintained, prevented, or slowed degradation of the electrical conduction in a subject's heart. The use of a holter monitor, a portable ECG

that can be worn for long periods of time to monitor heart abnormalities, arrhythmia disorders, and the like, is also a reliable method to assess the effectiveness of therapy.

Electrophysiology tests which involve percutaneous placement of catheters within the heart to assess the conduction properties of the heart, can also be used to assess therapy.

5 Where the condition to be treated is congestive heart failure, an echocardiogram or nuclear study can be used to determine improvement in ventricular function. Comparison of echocardiograms prior to and after the grafting of recombinant connexin cells into myocardial tissue allows for reliable assessment of treatment.

10 The above methods for assessing the efficacy of therapy are only exemplary and are not meant to be limiting. Many appropriate assays for detecting synchronized coupling, (e.g., by monitoring cardiac function) are well known in the art and can be adapted for use.

#### EXAMPLES

15 The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

20 While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

Example 1: Characterization of skeletal myoblasts/myotubes ability to electrically excite cardiac tissue.

Tissue engineering techniques are attractive alternatives to conventional therapies for the treatment of end stage heart disease and conduction abnormalities. Cell transplantation 5 offers the promise of restoring function to patients.

Biopsied skeletal muscle have satellite cells, skeletal myoblasts, which are able to divide and multiply. Skeletal myoblasts initially express Cx43. However, as the cells mature and differentiate into myotubes (the basic unit which leads to the contractile muscle fiber), Cx43 expression is the least in the skeletal myotubes.

10 Skeletal myoblasts and myotubes have different cellular electrophysiological characteristics. Characterization of the action potential parameters during different periods of myoblasts differentiation to myotubes were determined. Skeletal myoblasts were isolated by enzymatic dispersion from the hind limb muscle of 2-5 day old neonatal rats. Myoblasts were differentiated into multinucleated myotubes in culture by replacing the growth medium 15 with differential medium (DM). (98% DMEM, 2% horse serum (HyClone), penicillin G 100U/ml and streptomycin 100 $\mu$ g/ml). Myoblasts and myotubes incubated in DM 2-14 days were studied. Whole cell configuration of patch clamp technique was used to record action potentials. The following measurements were obtained: resting membrane potential (RMP), action potential amplitude (APA), action potential duration at 50 % (APD<sub>50</sub>) repolarization 20 (Table 1).

Myoblasts began to differentiate into multinucleated myotubes in 4 days and form a network of spontaneously contractile fibers by 10-14 days.

Table 1. Change of action potential parameters during different days in DM

25

group	RMP (mV)	APA (mV)	Vmax (V/s)	Threshold (nA)	APD <sub>50</sub> (ms)
DM 2 (n=10)	-27.4 $\pm$ 2.9	60.1 $\pm$ 5.8	27.4 $\pm$ 4.2	31.1 $\pm$ 6.5	15.1 $\pm$ 1.7
DM 4 (n=4)	-38.3 $\pm$ 3.6	94.4 $\pm$ 6.5	72.2 $\pm$ 6.7	23.8 $\pm$ 2.39	8.1 $\pm$ 0.1
DM 6 (n=8)	-50.6 $\pm$ 3.0	113.9 $\pm$ 3.4	102.9 $\pm$ 9.0	18.1 $\pm$ 0.9	7.4 $\pm$ 0.5
DM 8 (n=11)	-52.8 $\pm$ 1.8	123.7 $\pm$ 4.3	123.5 $\pm$ 6.4	17.2 $\pm$ 1.9	7.4 $\pm$ 0.4
DM 10 (n=7)	-53.1 $\pm$ 2.5	133.1 $\pm$ 2.7	153.4 $\pm$ 8.9	29.2 $\pm$ 5.3	7.4 $\pm$ 0.5
DM 11 (n=10)	-53 $\pm$ 1.7	133.5 $\pm$ 3.1	146.4 $\pm$ 2.9	33 $\pm$ 2.8	5.1 $\pm$ 0.5
DM 12 (n=9)	-52.4 $\pm$ 3.2	127.4 $\pm$ 2.9	142.7 $\pm$ 6.4	30.7 $\pm$ 6.8	5.4 $\pm$ 0.7
DM 13 (n=9)	-48.8 $\pm$ 3.1	120.6 $\pm$ 3.0	129.3 $\pm$ 6.8	30 $\pm$ 4.9	7.5 $\pm$ 0.6
DM 14 (n=9)	-46.8 $\pm$ 0.9	120.2 $\pm$ 5.4	114.6 $\pm$ 3.9	43.3 $\pm$ 4.3	6.4 $\pm$ 0.6

Freshly isolated skeletal myoblasts did not have measurable action potentials and were unable to be electrically stimulated.

**RMP.** There was no significant difference between days 8 and days 10-14 (FIG. 1A).

**APA:** With the RMP of myotube becoming more negative during development, the 5 amplitude of action potential also increased and reached to a peak value at 10-11 days. Then, APA decreased in parallel until 13-14 days. No significant difference was found between day 10 and day 8, day11-14. (FIG. 1B)

**V<sub>max</sub>:** similar changes were noticed as that of APA. (FIG. 1C)

**APD<sub>50</sub>:** The minium value of APD<sub>50</sub> occurred at 11-12 days and then increased. 10 There was no significant among each group except day 2. (FIG. 1D).

Thus, action potential parameters change during different periods of myoblasts differentiation to myotubes.

The patch clamping data highlights the relative electrical inexcitability of myoblasts in DM less than 7 days. The implications of these results is that transplanted skeletal 15 myoblasts/myotubes will not propagate an electrical impulse unless there is enhanced cellular coupling via gap junctions.

Computer modeling was used to assess cell to cell electrical excitation between skeletal myoblasts and myofibers with cardiac myocardial cells (Lee R et al., Annals of Biomedical Engineering 28-1:S54, 2000). The modeling was performed by incorporating 20 measured cellular parameters of each cell's type. The computer modeling results determined that the action potential duration (APD) of skeletal cells is short (1.6 ms and 2.8 ms for myoblast and myofiber, respectively), as compared to the cardiac cell, and is the major limitation of skeletal-to-skeletal and skeletal-to-cardiac excitation. A high degree of 25 intercellular coupling was required for skeletal cells to excite their downstream neighbors quickly enough, within 2.5 ms, prior to their own repolarization. The cardiac APD is long (178 ms) and there was a long length of time for cardiac cells to charge their downstream neighbor, before the charging cell repolarizes. Decreasing intercellular coupling increased the time necessary to charge adjoining cells. The ratio of intercellular coupling reduction to 30 still allow cell-to-cell excitation in homogeneous strands was 45:5:1 for the ventricular, skeletal myoblast, and skeletal myofiber cell types, respectively. In mixed strands, the limiting factor in excitation was any instance that the skeletal cell was the source cell.

These results demonstrate that: 1) the short skeletal action potential limits skeletal to cardiac conduction by limiting the capacity to provide a sufficient excitation charge to

cardiac cells; 2) skeletal myoblast differentiation into myofibers further limits excitation capacity; 3) very high levels of gap-junction coupling are needed for successful skeletal to cardiac conduction.

Thus, conditions which decrease intercellular coupling will markedly decrease  
5 electrical transmission between transplanted skeletal cells and the adjoining myocardium. Electrical conduction slowing or block can lead to potential life threatening arrhythmias.

Example 2 : Electrophysiologic consequences of skeletal muscle transplantation

To assess the electrophysiologic consequences of skeletal muscle transplantation into  
10 the myocardium, we utilized an in vivo model to assess cardiac conduction. The feasibility of gene transfer to specific areas of the cardiac conduction system has been previously demonstrated (Lee et al. 1998 PACE 21-II: 606; Gallinghouse et al. November 1996 Am Heart Assoc.; U.S. Patent No. 6,059,726). For example, the highly efficient and specifically localized expression of recombinant beta galactosidase in the AV node of rats and pigs has  
15 been described. The accuracy and reproducibility of AV nodal injections has been validated by the production of AV block in rats (Lee et al. 1998 J Appl Physiol. 85(2): 758-763). As an electrically insulated conduit for electrical transmission between the atrium and the ventricle, the AV conduction axis is in a strategic position for the study of cardiac electrophysiology.

20 To determine whether skeletal muscle transplantation alters conduction on AV nodal electrophysiologic properties, a rat model for AV node injections was utilized (Lee et al. 1998 J Appl Physiol. 85(2): 758-763). Animals were chemically denervated (using atropine and propranolol to inhibit the influence of autonomic nervous system) and studied with right atrial overdrive pacing and atrial programmed extrastimulation, both pre-injection and at the  
25 time of sacrifice. Surface ECG PR intervals were measured, together with AV nodal block cycle length (AVBCL) (the rate at which AV conduction becomes sequentially longer, then fails to conduct) and effective refractory period (ERP) (the coupling interval at which an atrial extrastimulus fails to conduct through the AV node). A single injection of skeletal myoblasts ( $1 \times 10^5$ , 15  $\mu$ l) or vehicle was injected into the AVN of rats (n=8).

30 Electrophysiologic properties of the AV junction were significantly altered in animals with transplantation of skeletal myoblasts. Significant alterations in the Wenkebach cycle length ( $70.0 \pm 4.4$  vs  $57.0 \pm 5.0$  msec;  $p < 0.01$ ) and AV nodal refractory period ( $113.8 \pm 5.6$  vs  $87.0 \pm 6.2$  msec;  $p < 0.005$ ) were recorded in the skeletal myoblast injected rats as compared to control animals. Histological examination of the AVN revealed that

approximately 10% of the AVN was involved with minimal to no inflammation.

Histologically the AV conduction axis appeared normal in control vehicle injections.

Interestingly, the PR interval did not significantly change, reflecting the insensitivity of surface EKG markers for cardiac conduction properties.

5 These results add further evidence that transplanted skeletal myoblasts (even when involving a small portion of the AVN) alters cardiac conduction and may lead to areas of slow conduction or conduction block. Therefore, as the skeletal myoblasts differentiate into myotubes and lose their ability to form gap junctions, the ability to propagate electrical impulses decrease.

10

#### Methods and Materials

The following materials and methods were utilized for Examples 3-7.

#### Skeletal myoblast isolation and culture.

15 This protocol was approved by the Committee on Animal Research, University of California at San Francisco and conducted in accordance with federal guidelines. Neonatal skeletal myoblasts were isolated as previously described by enzymatic dispersion from 2-5 days old C3H neonatal mice and cultured as previously described (Rando, T., and Blau, H. M. (1994), *J. Cell Biol.* 125, 1275-1287). After isolation, cells were cultured with growth medium 20 (GM) (80% F-10 medium (GIBCO BRL), 20% FBS (HyClone Laboratories, Inc.), penicillin G 100U/ml and streptomycin 100ug/ml, bFGF 2.5ng/ml(human, Promega Corp)). Skeletal myoblasts were maintained in GM medium in humidified 95% air and 5% CO<sub>2</sub>. Once the cultures achieved 75% confluence (day 0), the myoblasts were cultured in either GM medium or changed to differential medium (DM) (98% DMEM, 2% horse serum (HyClone), penicillin G 100U/ml and streptomycin 100ug/ml). Myoblasts cultured in DM were 25 incubated in humidified 95% air and 10% CO<sub>2</sub>. Myoblasts were collected on day 0, day 2, day 4, day 7, respectively for extraction of RNA and protein.

#### Production of Connexin 43

30 The rat connexin 43 (Cx43) cDNA was cloned into the MFG retroviral vector; and transduced into murine myoblasts as previously described (Springer ML, Chen AS, Kraft PE, Bednarski M, Blau HM., *Molecular Cell.* 1998, 2:549-558). This vector has been shown to be stably expressed in muscle (Dhawan J, Pan LC, Pavlath GK, Travis MA, Lanctot AM, Blau HM, *Science* 1991;254,1509-1512). Primary myoblasts already

expressing the *E. coli* β-galactosidase (β-gal) gene (TR/Z) was used as control myoblasts (Springer, M. L., and Blau, H. M., Som. Cell Mol. Genet. 1997;23, 203-209).

Determination of mRNA levels using RT-PCR

5 RNA from the cultured cells was prepared using the Qiagen Kit, Qiagen, Inc. CA, and quantified by spectrophotometry (A260 and A280 measurements). RNA (1ug) of each sample was reverse-transcribed for 1 hour at 37°C using Olig-dT and the same amount of cDNA was amplified for connexin 43, myogenin, myoD, desmin and GAPDH, respectively. The different primers used in this study were described in Table 2. After denaturing at 94°C  
10 for 5 minutes, amplification was performed for certain cycles (94°C for 30", 55°C for 30" and 72°C for 30"), followed by 72 cycles for another 5 minutes. The optimal cycles to semi-quantify the product for GAPDH and connexin 43 were 25; and for myogenin, myoD and desmin were 22. The PCR products were resolved by electrophoresis on 2% agrose gel and analyzed by densitometry with NIH software. The levels of connexin 43, myogenin, myoD,  
15 and desmin expression were normalized to the level of GAPDH; and the level of day 0 was set as 1.

**Table 2:** Summary of Primers utilized in Experimental studies:

Genes	Primer(Forward)	Primer(Reverse)
Connexin 43	5'- TACCACGCCACCACCGGCCCA -3'	5'- GGCATTGCTGTCGTCA GGGAA-3'
Myogenin	5'- CCTTAAAGCAGAGAGCATCC- 3'	5'- GGAATTCGAGGCATAATAT GA-3'
MyoD	5'- TTCTTCACCACACCTCTGACA- 3'	5'- GCCGTGAGAGTCGTCTTAA CTT-3'
Desmin	5'- CCGGAGGCTGGGTCGCT-3'	5'- CTGTTCTGAAGCTGGGCC TGG-3'
GAPDH	5'- AAAGTGGAGATTGTTGCCAT- 3'	5'- TTGACTGTGCCGTTGAATT- 3'

20

Detection of protein expression with Western blotting

The total soluble protein was extracted from the cultured cells and was quantified by Bradford method. The soluble proteins (40μg) were separated via SDS-PAGE using a 10-20% resolving gel for connexin 43, MHC, P21 detection. Proteins were electroblotted to HYBOND™-ECL nitrocellulose membrane and immunoreactions were carried out as

described using the ECL detection kit. Connexin 43 was detected using as anti-connexin 43 rabbit polyclonal antibody (Zymed Laboratories, Inc. Ca.) (1:1000). Myosin heavy chain protein was detected with Mf-20 antibody (Developmental studies hybridoma bank, University of Iowa) (1:2000 dilution). P21 protein was detected with P21 antibody (Chemicon international, Inc. Cz.) (1:500 dilution).

Immunofluorescence analysis

Immunofluorescence method for connexin 43, MHC, Desmin were performed as described by Tomakidi P, Cheng H, Kohl A, Komposch G, Alonso A, Cell Tissue Res, 2000;301(2):323-327. Briefly, myoblasts were plated on chamber slides with GM medium. At 70-80% confluence, the medium was either maintained in GM or switched to DM. Cells were collected on day 0, day 2, day 4 and day 7. After fixation with 4% paraformaldehyde in PBS and post fixative permeabilization with 0.2% triton X-100/PBS, cells were blocked with 3% BSA for 1 hour and incubated with primary antibody at room temperature for 1 hour. After washing with PBS three times, FITC-conjugated secondary antibody were used for incubation 1 hour. The dilution for Desmin antibody (Sigma, St. Louis, Mo), connexin 43 (Zymed Laboratories, Inc. Ca.) and MF-20 (Developmental studies hybridoma bank, University of Iowa) were 1:100, 1:100 and 1: 50, respectively.

Microinjection technique

Gap junctions were evaluated by microinjecting cells with the gap junction permeable dye, Lucifer yellow (Molecular Probes, Or.). Microinjection was performed in: 1) control(TR/Z) and CX43 myoblasts at 70-80% confluency, 2) TR/Z and CX43 myotubes and 3) co-cultured adult rat cardiomyocytes (ARC) and adult skeletal myoblasts or myotubes. The dye solution was composed of 2% Lucifer yellow (gap junction permeable) and 1% tetramethylrhodamine-dextran (gap junction impermeable; Molecular Probes) in sterile distilled water. Microinjection was performed with Micromanipulator 5171, FemtoJet, Eppendorf by a pulse pressure of 80hpa of 0.3 second of duration through a 0.5±0.2µm tip micropipette (Femtotips, Eppendorf). Cultured cells were washed and the medium was replaced with phosphate-buffered saline (PBS) containing 10% FBS. Injections were done with Nikon TE300 Microscope with phase and fluorescence optics.

Example 3 : Expression of Gap Junction Proteins

Connexin 43-encoding nucleic acid was introduced into skeletal muscle cells as described above. The formation of functional gap junctions between recombinant Cx43-expressing myoblasts or recombinant Cx43-expressing myoblasts which have differentiated 5 into myotubes with other types of myoblasts or myotubes was evaluated. A control (TR/Z) myoblast cell, which expresses Cx43 initially and then down regulates Cx43 expression during differentiation into myotubes was utilized as a control for functional gap junctions and dye transfer in control myoblast but not in control myotubes.

In Figures 2A-2D, the Cx43 mRNA (FIGs. 2A and B) and protein changes (FIGs. 2C and 10 D) in control cells and Cx43 cells are shown. Figure 2A is a photograph of an electrophoresis agarose gel of RT-PCR experiments indicating the mRNA Cx43 levels of control cells (TR/Z) and recombinant Cx43-expressing cells at day 0, 2, 4 and 7. Figure 2B is a graphical representation of the average level of Cx43 mRNA determined by RT-PCR for three control samples and three recombinant Cx43-expressing cell samples at day 0, 2, 4 and 7. FIG. 2A 15 and 2B show that the connexin 43 mRNA levels were significantly down-regulated by day 7 in TR/Z control (untransformed) skeletal myotubes while in contrast, the Cx43-modified cells exhibited no significant difference in Cx43 mRNA expression between day 0 and day 7, indicating that retroviral transduction with the connexin 43 gene was accomplished and Cx43 was expressed in mature myotubes unlike control myotubes (Day 7).

Figures 2C and 2D show the Cx43 protein levels associated with the same cells 20 analyzed for Cx43 mRNA depicted in FIG. 2A and 2B. Figure 2C is a photograph of a western blot for Cx43 protein, which indicates the relative amounts of Cx43 protein present in control cells and recombinant Cx43-expressing cells at day 0, 2, 4 and 7. Figure 2D is a graphical representation of Cx43 western blotting experiments to determine the relative 25 amount of Cx43 protein in three control cell samples and three Cx43 expressing cell samples at day 0, 2, 4 and 7. Protein expression results were consistent with the RT-PCR results confirming that expression of recombinant Cx43 can rescue connexin 43 loss in control cells at day 7 (FIG. 2C and 2D). The RT-PCR results shown in Figure 2A and 2B, demonstrate that Cx43 mRNA levels as expected, in control cells were gradually down and almost absent 30 at day 7 while the level of Cx43 mRNA for recombinant CX43 expressing cells was unchanged through day 0 to day 7. GAPDH was utilized as an internal control in these RT-PCR studies. Western blotting with antibodies for Cx43, in control cells showed that CX43 expression was downregulated at day 2 and almost absent after day 4 (during myotube formation) while recombinant Cx43-expressing cells did not show any downregulation, and

even upregulation could be detected at day 7. No differences in N-cadherin mRNA and protein expression levels were found in skeletal myoblasts before or after differentiation.

Microinjection studies to investigate the formation of functional gap junctions were completed on control cells (myoblast and myotubes) and recombinant Cx43-expressing cells (myoblasts and myotubes). Injected cells were labeled with rhodamine dextran and Lucifer Yellow, Lucifer yellow being capable of transfer from one cell to another through functional gap junctions. The black arrow in the phase contrast panels of Figure 3 indicates the injected cell in each set of experiments. Figure 3 shows photographs of a microinjection study between skeletal myoblasts or myotubes indicating the relative transfer of Rhodamine or Lucifer yellow dyes. Each panel of Figure 3 shows the cells of interest under phase contrast microscopy and appropriate fluorescence illumination for either Rhodamine or Lucifer yellow fluorescent dyes, FIG 3 row A is a photograph of control myoblasts which express Cx43, contacting other control myoblasts; row B is a photograph of Cx43 myoblasts to Cx43 myoblasts; row C shows control myotube (no Cx43 expression) to control myotube and row D shows photographs of Cx43 myotube to Cx43 myotubes.

These microinjection studies show that in skeletal myoblasts, dye transfer (Lucifer yellow) could be observed in both control (TR/Z) and Cx43 myoblasts (FIG 3, :rows A and B). After 7 days in culture with DM media, no dye transfer could be observed in myotubes formed from control myoblasts, FIG 3, :row C. Dye transfer persisted in Cx 43 transduced skeletal cells placed in differentiation media for 7 days (FIG. 3, :row D). In summary, these microinjection experiments showed that dye transfer occurred in Cx43 transduced skeletal myoblasts placed in differentiation media and not in control myotubes.

#### Example 4 : Gap Junction Function and co-culture experiments

To evaluate gap junction formation between myoblasts and cultured adult rat cardiomyocytes (ARC), single adult rat cardiac ventricular myocytes were enzymatically isolated from female Sprague-Dawley rats weighing 200-250g by standard methods. Briefly, following intraperitoneal anesthesia (pentobarbital 100mg/kg), the rat heart was rapidly excised and perfused retrogradely via the aorta using the Langendorff technique. The perfusion was performed at 37° C using solution A (nominal Ca<sup>2+</sup> free solution, NaCl 134 mM, KCl 5.4 mM, Hepes 10 mM, glucose 10mM, MgCl<sub>2</sub> 1 mM, NaH<sub>2</sub>PO<sub>4</sub> 0.33 mM, titrated to pH 7.4 with NaOH.) for 5 min, solution A, 0.1 mM CaCl<sub>2</sub> with 1 mg/ml collagenase (Type B, Boehringer Mannheim, Germany) for about 15 min consequently, then washout with solution A and CaCl<sub>2</sub> 0.2 mM for 5 min. Afterwards the left ventricle was

removed and chopped into small pieces, which were incubated with 20 ml solution A and 0.1 mM CaCl<sub>2</sub> with shaking at 37°C for 10 min in a glass conical flask. The cell suspension was filtered (200 micron mesh) and the filtrate was sedimented for 5 min. The Ca<sup>2+</sup> concentration of the supernatant was gradually increased with 1mM Ca<sup>2+</sup>-containing solution till 0.5 mM final concentration. ARC were grown in HAM-F-12/M199 (1:1) supplemented with 10% FBS, penicillin G 100U/ml and streptomycin 100µg/ml in laminin-coated dishes at densities of 10<sup>4</sup> rod-shaped cells cm<sup>-2</sup>.

In serum-containing medium, ARC undergo a morphological change described as dedifferentiation/redifferentiation, hallmarked by the loss of the rod shape and myofibrillar disintegration and subsequent spreading, and reorganization of the contractile apparatus. On day 3, cytosine arabinouranoside (5 µM) was added to prevent fibroblasts overgrowth. Most of the ARC were redifferentiated by day 7 and contractile activity was observed. After completion of differentiation/redifferentiation, skeletal myoblasts (10<sup>4</sup>/cm<sup>2</sup>) were added to the ARC cultures. They were kept in the HAM-F-12/M199 medium for overnight and microinjection was performed next day to evaluate dye transfer between myoblast and ARC. To induce myotubes formation, the medium was changed to DM and microinjection was performed after myotubes formation (7 days).

Microinjection studies to investigate the formation of functional gap junctions between cardiomyocyte cells and control cells (skeletal myoblast and myotubes) or with recombinant Cx43-expressing cells (skeletal myoblasts and myotubes) were completed. Injected cells were labeled with rhodamine dextran and Lucifer Yellow, Lucifer yellow being capable of transfer from one cell to another through functional gap junctions. The black arrow in Figure 4 indicates the injected cell in each set of experiments. In co-culture experiments, dye transfer could be observed between adult rat cardiomyocytes (ARC) and control myoblast (which express Cx43, FIG. 4, row A) or with Cx43 myoblasts (FIG. 4, row B). Even after 7 days in differentiation culture, Cx43 cells were capable of dye transfer with ARC, indicating functional gap junctions(FIG 4, row D). In contrast, there was no dye transfer between control skeletal myotubes and ARC as shown in Figure 4, row C. In summary, these experiments indicate the unique and novel features of the present invention by demonstrating that it is possible to form functional gap junctions between two different cell types by expressing a recombinant connexin in one of the cells. In particular, that functional gap junctions can be formed between adult skeletal muscle cells modified to over express Cx43 and cardiomyocytes.

Example 5: Effects of Connexin 43 expression on skeletal myoblasts differentiation

To determine the effect of Cx 43 expression on the differentiation of skeletal myoblasts, expression levels of other proteins were analyzed. Figure 5 shows the results from an Immunofluorescence study analyzing the expression levels of MHC and Desmin, two strong markers for myoblast differentiation into myotubes, in control and Cx43 cells (MHC:FIG. 5, upper panel and Desmin:FIG. 5, lower panel). Control skeletal myoblasts differentiated into multinucleated myotubes after incubation with DM for 7 days. In the Connexin 43 group, myotubes did not form even after 14 days in DM. Clearly, expression of recombinant Cx43 prevented myoblasts from forming myotubes. Immunofluorescence studies shown in Figure 5 demonstrate that MF-20 (MHC) and Desmin, two strong markers for myoblast differentiation into myotubes, were present at day 7 in control samples and absent in the CX 43 expressing samples. The MHC and Desmin results are shown in the upper and lower panels of FIG. 5, respectively. MF-20 expression from western blotting study was consistent with immunofluorescent study. P21 expression, marker of cell mitosis arrest, had consistent changes among these groups and was up-regulated gradually from day 0 to day 7, which reflects that both TR/Z and Cx43 cells withdraw from dividing when medium was switched to DM.

To determine whether the expression of recombinant connexin 43 is harmful to myotubes or is only deleterious during differentiation from myoblasts to myotubes, skeletal myoblasts and myotubes were transfected with a replication-deficient adenovirus with the Cx43 gene (Ad Cx43). Myoblasts transfected with Ad Cx43 and transferred to differentiation media had impaired myotube formation. In contrast, fully differentiated myotubes transfected with Ad Cx43 remained normal appearing and aligned themselves in an orderly array analogous to control myotubes. Transfection with control adenovirus without Cx43 developed normally.

Example 6: Cx 43 expression in skeletal muscle improves electrical conduction in the AV node.

To determine whether the forced expression of connexins improve cardiac conduction, skeletal muscle cells were transduced with Cx 43 (same cells as used in in vitro experiments) and injected into the AV node of immunodeficient rats (Lee et al. 1998 J Appl Physiol. 85(2): 758-763). Animals injected with Cx 43 transduced skeletal myoblasts ( $2.5 \times 10^6$  cells/ 25  $\mu$ l; n= 8) were compared to animals injected with control skeletal myoblasts ( $2.5 \times 10^6$  cells/ 25  $\mu$ l; n= 5). Surface ECG PR intervals were measured, together with AV

nodal block cycle length (AVBCL) (the rate at which AV conduction becomes sequentially longer, then fails to conduct) and AVN effective refractory period (AVN ERP) (the coupling interval at which an atrial extrastimulus fails to conduct through the AV node).

Significant shortening of the PR interval was observed in the animals injected with  
5 Cx43 transduced skeletal myoblasts as compared to the control skeletal muscle cell injected  
animals ( $40.6 \pm 1.9$  ms vs  $47.6 \pm 2.5$  ms;  $p < 0.0001$ , paired T-test). The AVBCL ( $96.7 \pm 10$   
ms vs  $112.0 \pm 11.0$  ms;  $p < 0.03$ , paired T-test) and AVN ERP ( $80.0 \pm 9.2$  ms vs  $100.0 \pm$   
 $16.0$  ms;  $p < 0.001$ , paired T-test) were significantly improved in animals injected with Cx43  
transduced skeletal myoblasts as compared to animals injected with control skeletal  
10 myoblast

These results demonstrate that the electrical conduction through the AV junction was  
significantly improved in animals injected with Cx43 transduced skeletal myoblasts as  
compared to control skeletal myoblasts. Thus connexin production in the recombinant cells  
provided for electrical connection between the recombinant cells and adjoining myocardial  
15 cells, which in turn would provide for better electromechanical synchrony between the atria  
and the ventricle.

Example 7 : Autologous Transplantation of Cx43-Expressing Cells in Patients with a  
Previous Myocardial Infarction

20 The treatment of cardiomyopathy in humans is carried out as follows. A muscle  
biopsy is obtained from patients who have experienced anterior, lateral or inferior wall  
myocardial infarction and may or may not be a patient that requires coronary artery bypass  
graft (CABG) surgery. The skeletal muscle cells gathered from the biopsy are cultured *ex*  
*vivo* and genetically modified to express a human connexin (such as Cx43) by the methods  
25 described above. The modified skeletal muscles are analyzed for recombinant connexin  
expression by immunofluorescence assay for connexin protein. In certain instances, the cells  
are analyzed for the ability to form functional gap junctions with cardiomyocyte cells by the  
*in vitro* Lucifer dye assays described above.

After analysis of the modified muscle cells, a therapeutically effective amount of the  
30 modified muscle cells are implanted into the patients heart tissue. In certain instances when  
the patients own skeletal muscle cells cannot be used for cardiac treatment, a recombinant  
muscle cell line which expresses recombinant human Cx43 is utilized in conjunction with  
the appropriate use of immunosuppression drugs known to those skilled in the art. The Cx43  
expressing muscle cells are then implanted endovascularly with a injection catheter, which

catheters can be obtained from a variety of sources (e.g., injectable catheters such as Johnson & Johnson's NOGA system, BioHeart's Myocath, Biocardia, Boston Scientific's stiletto, Transvascular catheter, and the like) or with a hypodermic syringe for a CABG procedure. The patient is monitored after surgery to evaluate the efficacy of treatment.

5

*Patients*

The patients are males and females generally between 18 and 75 years of age with the diagnosis of previous myocardial infarction or non-ischemic cardiomyopathy.

10

*Biopsy*

The skeletal muscle biopsy is obtained within a few weeks (e.g., 3-4 weeks) of anticipated coronary artery bypass for patient where the procedure is warranted. Autologous skeletal muscle cells (myoblasts and myotubes) are isolated from the skeletal muscle biopsy. Under sterile surgical conditions, an open biopsy technique is utilized to excise skeletal muscle from the muscle belly. The biopsy is obtained from the thigh (Quadriceps-vastus lateralis) or the mid-calf (Gastrocnemius) of the patient. An attempt is made to exclude contaminating fascia from the biopsy.

Quadriceps-vastus lateralis- An incision is made longitudinally along the anterolateral aspect of the thigh in the lower third of the thigh. Dissection is carried through the soft tissue and fascia and the quadriceps vastus lateralis will be identified and exposed. A segment of muscle is resected longitudinally along the long axis of the muscle fiber and placed into a container of transport medium.

Gastrocnemius- An incision is longitudinally in the posterolateral gastrocnemius area in the mid calf. Dissection is made through to the deep fascia to expose the gastrocnemius muscle. A segment of muscle is resected longitudinally along the long axis of the muscle fiber and placed into a container of transport medium.

*Ex vivo propagation and genetic modification of autologous cells*

The methods and protocols used for the isolation, expansion and transduction of the autologous skeletal muscle cells with a human connexin construct *ex vivo* are as described above. For example, human connexin (e.g., Cx43) cDNA is cloned into the MFG retroviral construct and transduced into the autologous skeletal muscle cells in a similar manner as described by Springer ML et. al., Molecular Cell. 1998, 2:549-558. This construct is generally stably expressed in the autologous muscle cells.

The genetically modified cells are cultured so as to provide for a concentration of about  $10^6$ - $10^9$  cells/ml. The modified cells may be stored under refrigeration (usually around 0°C) prior to transplantation into the patient. Cell viability via Trypan Blue Dye Exclusion can be used as a cell viability assay. Potency is confirmed via the detection of Cx43 expression by immunofluorescence and/or by the functional gap junction assays described above.

- Implantation of recombinant connexin expressing cells via a percutaneous approach*
- Implanting the recombinant connexin expressing cells into the myocardium involves 10 administering the recombinant cells by using a catheter delivery system. The recombinant cells are injected into the akinetic myocardial scar at the site of a previous infarct. Depending on the size of the targeted infarct zone, between 400 million and 1 billion cells are injected as a suspension. Multiple injections can be used to deliver the recombinant cells.
- 15 The injections are carried out by advancing the needle through the end hole of the catheter to a predetermined depth. The proximal end of the needle lumen is attached to a calibrated syringe that contains the recombinant cell suspension. After adequate positioning against the endocardial surface by fluoroscopic, intracardiac echocardiography or magnetic resonance imaging guidance, the needle is advanced into the myocardium and the cell suspension is injected. Upon completion of the injection, the needle is withdrawn into the catheter. This method is repeated in the target region until transfer of the cells is complete. An attempt is made to cover the entire area of the scar, including its periphery. If the cellular therapy is delivered during a CABG, then a needle and syringe are used to epicardially deliver the cells to the akinetic region as described above.

25

#### *Monitoring and Evaluation of Treatment*

Clinical status, adverse events, 12-lead electrocardiogram, 24 hour ambulatory electrocardiogram, and routine clinical laboratory tests are carried out by methods and techniques known to those skilled in the art for the evaluation of regional left ventricular wall function. Follow-up can be performed and compared to baseline (i.e., prior to treatment) at selected periods post-implantation (e.g., 1, 2, 3, 4, 6, and 12 months). In certain instances, evaluation of treatment may include Dobutamine stress echocardiographic evaluation of regional wall motion and wall thickness in region of implantation (infarcted region), contrast ventriculography or magnetic resonance imaging. The monitoring and

evaluation post treatment can be used to determine the level of regeneration of functional muscle and synchronized electromechanical conduction within the infarct.

Example 8: Autologous Transplantation of Recombinant Cx43-Expressing Cells in Patients

5 with cardiac conduction disease.

*Patients*

The patients are males and females between 1 and 90 years of age with the diagnosis of cardiac conduction disease (i.e., heart block). The heart block can be congenital, acquired, iatrogenic (e.g., as a complication of valve surgery or catheter ablation) or part of 10 the normal aging process. Utilizing the methods described in Example 7, 1 – 100 million modified cells can be injected in the AV node region in a volume of 0.2-0.5 ml. The recombinant connexin cells can be delivered surgically via a 25 gauge syringe, via the AV nodal artery or via a percutaneous delivery system (see, e.g., U.S. Patent No. 6,059,726.

15 *Monitoring and Evaluation of Treatment*

The detection of heart block (and its treatment) can be readily detected by surface ECG. Exercise stress testing, holter monitoring or an electrophysiology study are alternative supplemental tests to assess therapy.

**CLAIMS**

That which is claimed is:

1. A method of establishing an electrical connection between a recombinant mammalian cell and a myocardial cell, the method comprising:
  - 5 contacting a myocardial cell with a recombinant mammalian cell genetically modified to produce a connexin protein, said contacting being in a manner sufficient to provide for production of an electrical connection between the myocardial cell and the recombinant cell;
  - 10 wherein an electrical connection between the recombinant cell and the myocardial cell is established.
2. The method of claim 1, wherein the recombinant cell is selected from the group consisting of a skeletal muscle cell, a stem cell, a fibroblast, and a cardiac cell.
  - 15 3. The method of claim 1, wherein the recombinant cell is a skeletal muscle cell.
  4. The method of claim 3, wherein the skeletal muscle cell is an adult skeletal muscle cell.
    - 20 5. The method of claim 3, wherein the skeletal muscle cell is a myoblast cell.
    6. The method of claim 1, wherein the connexin protein is a connexin 43 protein.
  - 25 7. A method of claim 1, wherein said contacting comprises implanting the recombinant cell into myocardial tissue of a subject.
  8. The method of claim 1, wherein after the electrical connection between the recombinant cell and the myocardial cell is established, the recombinant cell has similar conductive characteristics similar to the myocardial cell.
    - 30 9. A method of establishing an electrical connection between a recombinant skeletal muscle cell and a myocardial cell, the method comprising:

contacting a myocardial cell with a recombinant skeletal muscle cell genetically modified to express a connexin protein, said contacting being in a manner sufficient to provide for production of an electrical connection between the myocardial cell and the recombinant skeletal muscle cell;

5 wherein an electrical connection between the recombinant skeletal muscle cell and the myocardial cell is established.

10. The method of claim 9, wherein the skeletal muscle cell is an adult skeletal muscle cell.

10

11. The method of claim 9, wherein the skeletal muscle cell is a skeletal myoblast cell.

15

12. The method of claim 9, wherein after the electrical connection between the recombinant cell and the myocardial cell is established, the recombinant cell has similar conductive characteristics as the myocardial cell.

20

13. A method of establishing an electrical connection between a recombinant skeletal muscle cell and a myocardial cell, the method comprising:

contacting a myocardial cell with a recombinant skeletal myoblast cell genetically modified to express a recombinant connexin 43 protein, said contacting being in a manner sufficient to provide for production of an electrical connection between the myocardial cell and the recombinant skeletal myoblast cell;

25

wherein an electrical connection between the recombinant skeletal myoblast cell and the myocardial cell is established so that the recombinant skeletal myoblast cell has similar conductive characteristics as the myocardial cell.

14. A method for treating a cardiac conduction disturbance in a host, the method comprising:

30

introducing into cardiac tissue of a host a therapeutically effective amount of a recombinant mammalian cell, which recombinant cell is genetically modified to express a connexin protein, said introducing being effective to establish an electrical connection between the recombinant cell and a myocardial cell of the host cardiac tissue;

wherein the cardiac conduction disturbance in the host is treated.

15. The method of claim 14, wherein the recombinant cell is selected from the group consisting of a skeletal muscle cell, a stem cell, a fibroblast, and a cardiac cell.

16. The method of claim 14, wherein the recombinant cell is a skeletal muscle cell.

5

17. The method of claim 16, wherein the skeletal muscle cell is an adult skeletal muscle cell.

18. The method of claim 16, wherein the skeletal muscle cell is a myoblast cell.

10

19. The method of claim 14, wherein the connexin protein is a connexin 43 protein.

20. The method of claim 14, wherein said introducing comprises implanting the recombinant cell into an infarct region of the cardiac tissue.

15

21. The method of claim 14, wherein the recombinant cell is autologous to the host.

22. A method for treating a cardiac conduction disturbance in a mammalian host, the method comprising:

20

introducing into cardiac tissue of the host a therapeutically effective amount of a skeletal muscle cell genetically modified to express a connexin 43 protein, said introducing being effective to establish an electrical connection between the introduced recombinant skeletal muscle cell and a myocardial cell of the host cardiac tissue;

wherein the cardiac conduction disturbance is treated.

25

23. The method of claim 22, wherein the skeletal muscle cell is an adult skeletal muscle cell.

30

24. A method of claim 22, wherein the skeletal muscle cell is a myoblast cell.

25. The method of claim 22, wherein said introducing comprises implanting the recombinant cell into an infarct region of the cardiac tissue.

26. The method of claim 22, wherein the recombinant skeletal muscle cell is autologus to the host.

1 / 7

FIG. 1A

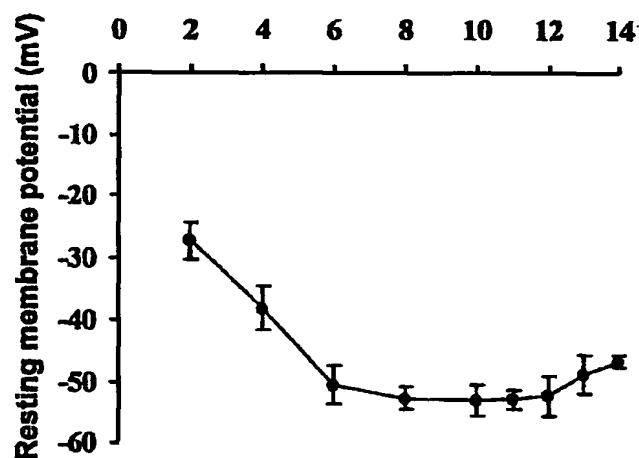
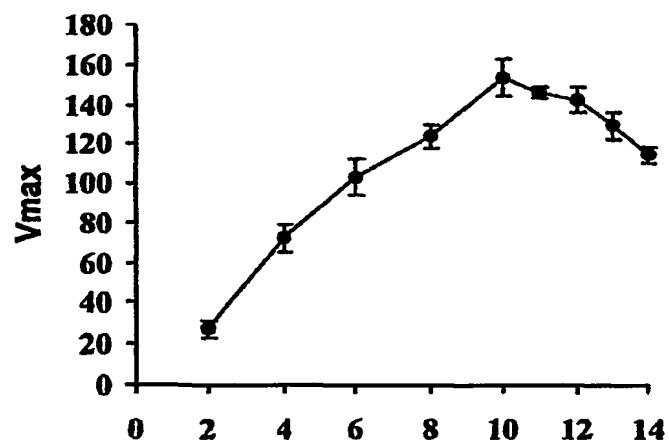


FIG. 1C



2 / 7

FIG. 1B

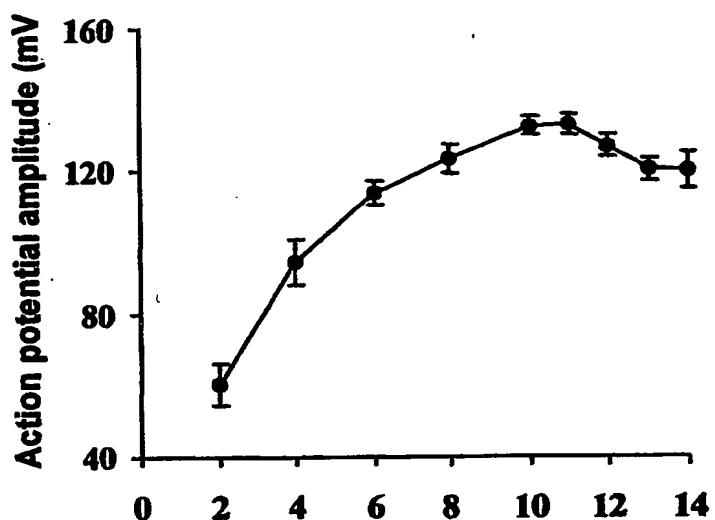
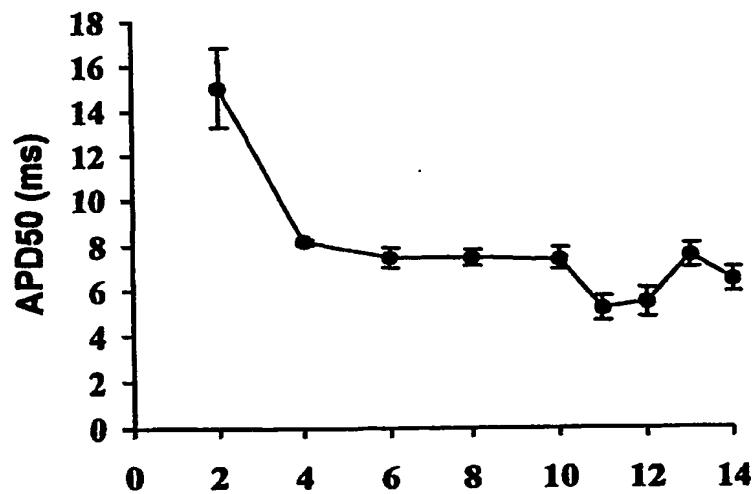
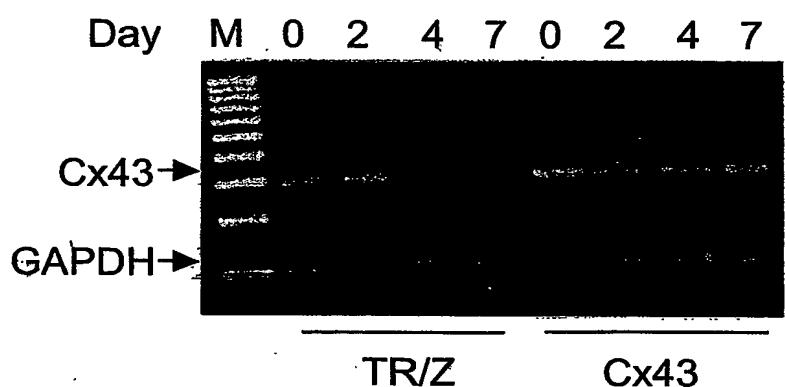
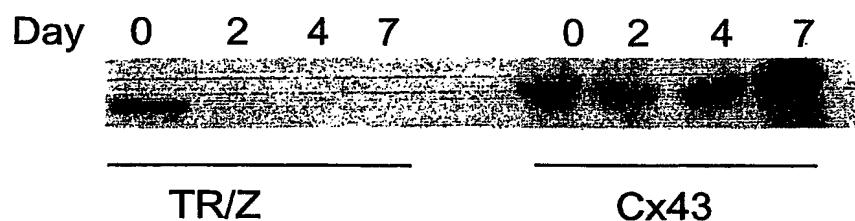


FIG. 1D



3 / 7

**FIG. 2A****FIG. 2C**

4 / 7

FIG. 2B

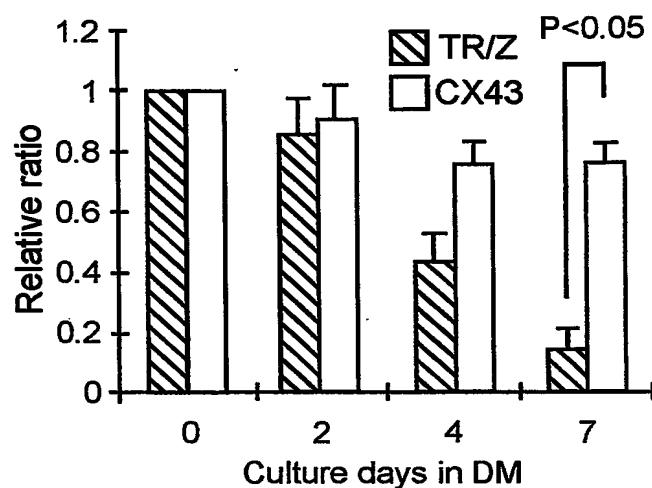
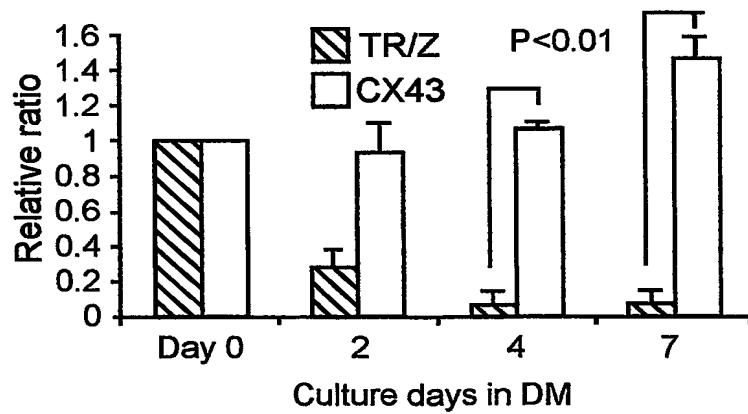


FIG. 2D



5 / 7

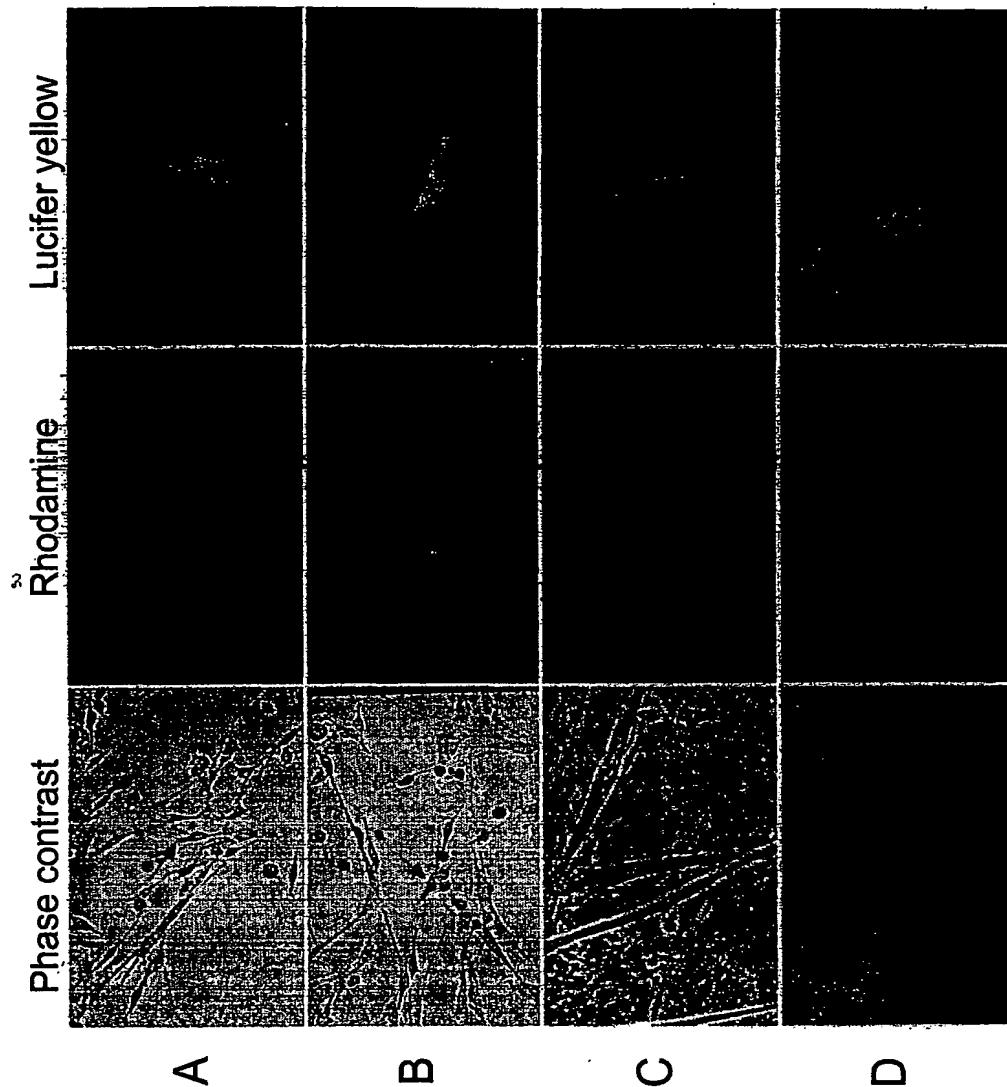


FIG. 3

6 / 7

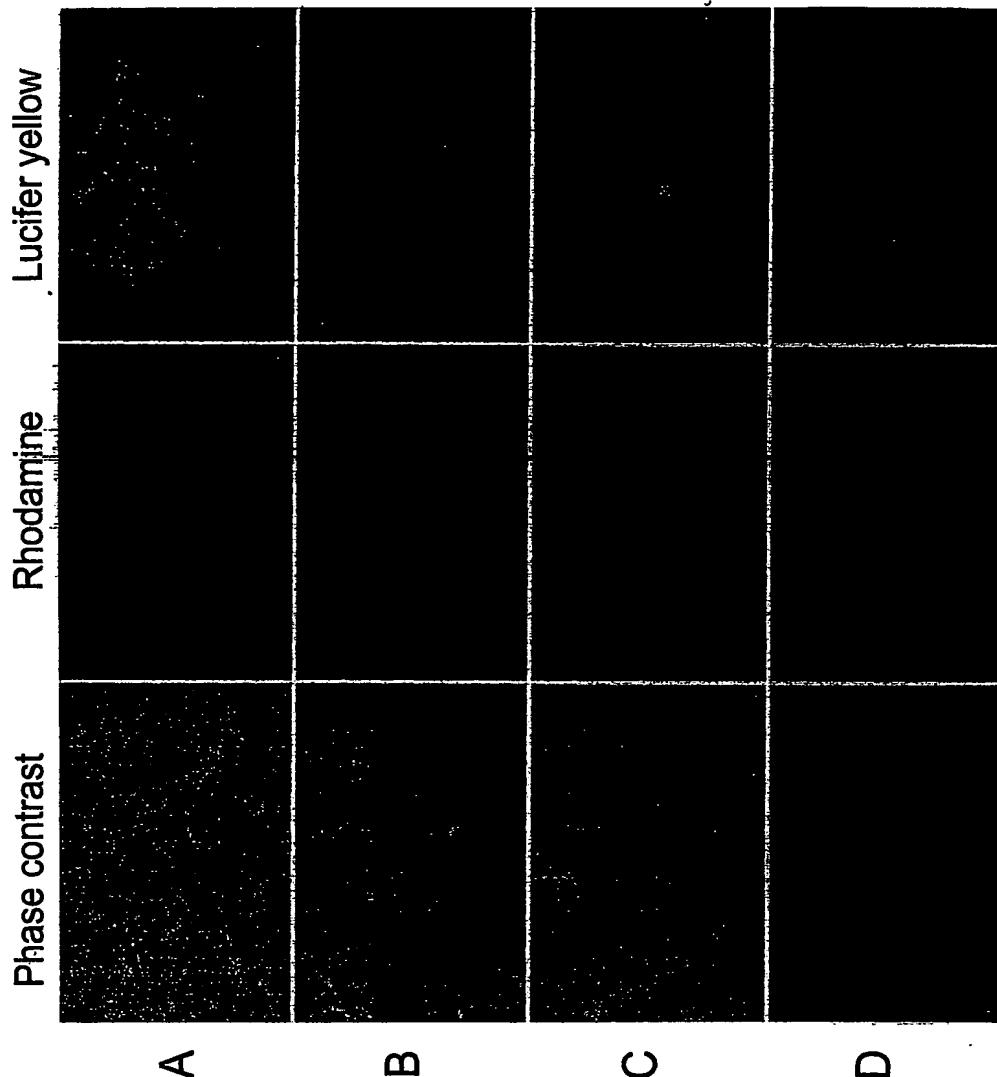


FIG. 4

7 / 7

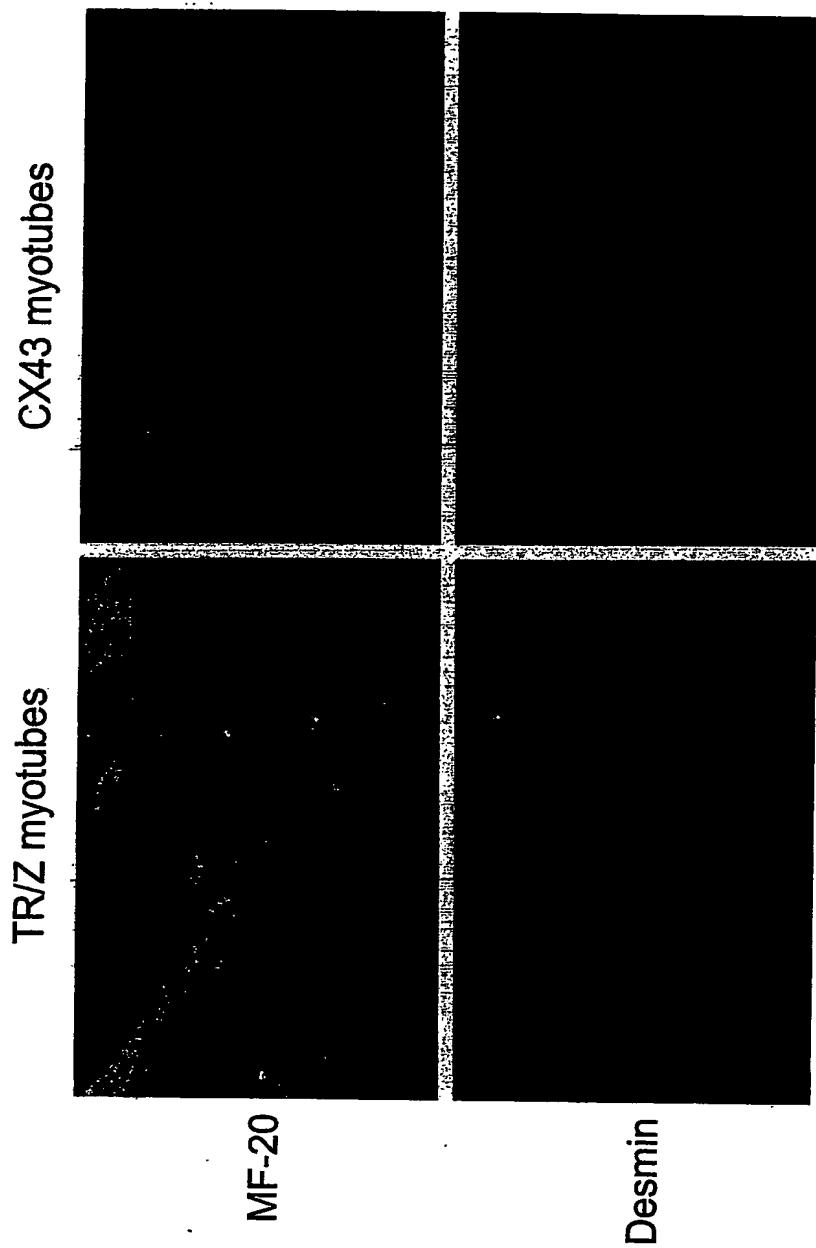


FIG. 5

## (12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization International Bureau



(43) International Publication Date  
20 November 2003 (20.11.2003)

PCT

(10) International Publication Number  
**WO 2003/094697 A3**

- (51) International Patent Classification<sup>7</sup>: **A61K 48/00**
- (21) International Application Number:  
**PCT/US2003/014304**
- (22) International Filing Date: **7 May 2003 (07.05.2003)**
- (25) Filing Language: **English**
- (26) Publication Language: **English**
- (30) Priority Data:
- |                |                              |    |
|----------------|------------------------------|----|
| 60/379,140     | 8 May 2002 (08.05.2002)      | US |
| 10/291,202     | 7 November 2002 (07.11.2002) | US |
| PCT/US02/35932 | 7 November 2002 (07.11.2002) | US |
- (71) Applicant (*for all designated States except US*): **THE REGENTS OF THE UNIVERSITY OF CALIFORNIA**  
[US/US]; 1111 Franklin Street, 12th Floor, Oakland, CA 94604-5200 (US).
- (72) Inventor; and
- (75) Inventor/Applicant (*for US only*): **LEE, Randall, J.** [US/US]; University of California, Cardiac Electrophysiology, Rm. MU429, 500 Parnassus Avenue, San Francisco, CA 94143 (US).
- (74) Agent: **FRANCIS, Carol, L.; Bozicevic, Field & Francis LLP**, Suite 200, 200 Middlefield Road, Menlo Park, CA 94025 (US).
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

## Published:

- *with international search report*
- *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report:  
**27 May 2004**

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 2003/094697 A3

## (54) Title: METHODS AND COMPOSITIONS FOR CORRECTION OF CARDIAC CONDUCTION DISTURBANCES

(57) Abstract: The invention provides methods for establishing electrical coupling between cardiomyocytes and recombinant cells which have been genetically engineered to express a gap junction protein, e.g., a connexin protein such as connexin 43 (Cx43) protein. The invention is based on the discovery that genetic modification of skeletal muscle cells to express a recombinant connexin, enables the genetically modified cells to establish electrocommunication with cardiac cells via gap junctions. The recombinant connexin-expressing cells can be used for repair of cardiac tissue and for treatment of cardiac disease by transplantation into cardiac tissue.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/14304

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) : A61K 48/00  
 US CL : 424/93.21

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
 U.S. : 424/93.21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
 EAST, biosis, medline, caplus, embase**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2002/0001577 A1 (HAVERICH et al) 3 January 2002 (03.01.2002). See entire document.	1-26
A	NICHOLAS et al, Immunocytochemical analysis of connexin expression in the healthy and diseased cardiovascular system. Microscopy Res. Technique. 2001, Vol. 52, pages 301-322. See entire document.	1-26

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B" earlier application or patent published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Z"	document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

07 March 2004 (07.03.2004)

Date of mailing of the international search report

05 APR 2004

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US  
 Commissioner for Patents  
 P.O. Box 1450  
 Alexandria, Virginia 22313-1450  
 Facsimile No. (703)305-3230

Authorized officer

Q. Janice Li

Telephone No. 703-308-0196

*J.D.R. JYL*

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**